Pakistan J. Zool., pp 1-9, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20220919070950

Sero-Epidemiology and Evaluation of First Self-Prepared Bovine Viral Diarrhea Virus Vaccine in Cattle of Punjab, Pakistan

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Article Information
Received 19 September 2022
Revised 25 September 2022
Accepted 13 October 2022
Available online 14 June 2023
(early access)

Authors' Contribution
AA and MR designed and planned this study. QA carried out experiments and analyzed the data. FNA provided serum sample to investigate this study. HM revised the manuscript for important contents.

Key words
Sero-epidemiology, BVDV, Cattle, ELISA, Risk factors, Vaccine

ABSTRACT

This study was undertaken to determine the sero-epidemiology and the efficacy of the first self-prepared BVDV vaccine in cattle. In a cross sectional survey (2020–2021) conducted in 5 agro-climatic zones of Punjab, a total of 1000 serum samples from 100 dairy herds were collected and the sero-prevalence was estimated using an Ab capture ELISA assay. Inactivated BVDV vaccine was also prepared using local isolate (MK084980) and tested for induction of humoral immune response in comparison with commercial vaccine in experimental cattle calves. Calves of group A (n=5) and B (n=5) were vaccinated with self-prepared and commercial vaccines respectively followed by boosting 30 days post primary vaccination, while group C was kept as non-vaccinated control group. Blood samples were taken from each group after every 2 months up to one year post vaccination and tested through Ab capture ELISA. Overall, 23.7% sero-positivity of BVDV was observed in 5 agro-climatic zones with highest in the low-intensity zone (34%) followed by rice wheat (27%), cotton-wheat (21%), mixed-crop (19%) and lowest in the rain-fed zone (17.5%). Quarantine practices, housing management, abortion and parity were found the potential risk factors significantly associated ($p<0.05$) with BVDV prevalence while breeding system exhibited non-significant association with BVDV ($p>0.05$). In vaccine trial, self-prepared vaccine proved to be equally effective as commercial vaccine although higher ($p>0.05$) antibody titer (s/p values) were noted in group B as compared to group A. Throughout the experiment, antibody titer of vaccinated groups remained significantly higher ($p<0.05$) than group C. Both the vaccinated groups showed protective ELISA antibody titers (S/P value) at 4 months post vaccination that persisted up-to 10th months followed by decline indicated the need of revaccination on annual basis. This study first time confirmed the circulation of BVDV in all the 5 agro-climatic zones of Punjab and may be vaccinated preferably with self-prepared vaccine due to cost effectiveness and usage of local vaccinal virus.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is considered as one of the most significant pathogens causing reproductive disorder resulting in huge economic losses to the livestock industry. Other than reproductive disorders, BVDV produce lesions in GIT, respiratory, CNS, circulatory and integumentary systems as well (Elvira Partida *et al*., 2017; Silveira *et al*., 2017). BVDV (12.5 kb) is a member of pestivirus in the Flaviviridae family. It is an enveloped, positive polarity single stranded RNA virus (Lefkowitz *et al*., 2018). BVDV mainly infects the bovines but serological evidence of BVDV infection have been reported in more than forty different species (Colom-Cadena *et al*., 2016; Feknous *et al*., 2018). Major economic losses due to BVDV infection incur in the form of morbidity and mortality, reduced production and higher premature culling rate of infected animals (Scharnböck *et al*., 2018). Based

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0030-9923/2023/0001-0001 $ 9.00/0

on growth characters in cell culture, BVDV is divided into two biotypes; cytopathic (CP) and non-cytopathic (NCP). NCP strain is responsible for development of persistence in animals. Persistently infected (PI) animals shed the virus continuously throughout their life therefore considered as the major source of infection in cattle herds (Garoussi *et al*., 2019). If naive cattle get infected from acute BVDV infection, it may result in transient infection (TI), although seroconversions 3 weeks post infection confer lifelong immunity. Transmission of BVDV infection occurs via horizontal and vertical routes (Timurkan and Aydın, 2019). Seroprevalence of BVDV estimated in United Nations regions ranged from 46.23% to 48.73% at the animal level, and from 66.08% to 67.01% at the herd level (Scharnböck *et al*., 2018). Sero-positivity against BVDV confirmed in approximately 88 countries and mitigation activities reported in 107 countries between 1960 to 2017 (Richter *et al*., 2019). Prevalence variation in different regions and herds indicates the lack of implementation of preventive measures such as vaccination, quarantine practices and associated various risk factors that help the spread of virus (Aragaw *et al*., 2018). The countries with high rate of BVDV infection, vaccination is prioritized as mainstay tool for BVDV control along with other biosecurity measures. The key role of vaccination is the provision of cross protection against BVDV-1 and BVDV-2 that help prevent transplacental transfer of infection from dam to fetus thus limit the development of PI. Numerous studies have provided the substantial evidence of BVDV vaccine efficacy by lowering the incidence rate of BVDV infection in herds (Álvarez *et al*., 2012; Kurćubić *et al*., 2011; Stott *et al*., 2010). Due to high genetic diversity of BVDV both live attenuated and killed vaccines have been developed to control infection. Generally broader and long-lasting immunity is provided by live vaccines (Griebel, 2015). However, killed vaccines combined with an adjuvant tend to induce adequate immune response but for shorter duration (Elhelw *et al*., 2022). Therefore, revaccination of herd is required that increase vaccination cost. Although, key concern for BVDV vaccine, often raised is the development of protective immunity because of high antigenic dissimilarity of BVDV genotypes. Studies have indicated poor cross protection of one genotype strain from other genotype strains (Abd El-Fadeel *et al*., 2020). This is the most important aspect for vaccine development to be considered. In Pakistan, no vaccination strategy is in place possibly due to unavailability of true picture of BVDV. The major obstacles to adopt BVDV vaccination program is the lack of awareness among the livestock farmers and cost effectiveness of vaccines. In recent past a small-scale serological study reported 18.77% BVDV prevalence in southern Punjab, Pakistan (Raheem *et al*., 2020). Collectively, economic significance and available research data makes it imperative for further research to find out the actual status of BVDV in the country. Therefore, the purpose of present study was to estimate the BVDV status in the five agro climatic zones of Punjab and measurement of comparative protective efficacy of self-prepared vs commercial vaccine in experimental calves.

MATERIALS AND METHODS

For sero-epidemiology of BVDV, a cross sectional study was undertaken in the five agro climatic zones of Punjab including rice-wheat, mixed-crop, cotton-wheat, low intensity and rain-fed (Barani). The estimated sample size (n=1000) was determined assuming the 50% prevalence of BVDV in the study area (Thrusfield, 2018).

Of the 5 zones, a total of 1000 blood samples were randomly collected from 100 dairy herds. All the dairy herds were non-vaccinated for BVDV. From each zone, 4 districts were selected on the basis of livestock population (Livestock Census, 2018) and from each district 5 dairy farms were sampled (10 animals from each farm). A questionnaire was developed to glean the information of various risk factors: quarantine practice (yes/no), breeding system (artificial insemination/natural), housing management (intensive/extensive), abortion history (yes/no) and parity status (≤ 2/≥2). Information pertinent to various risk factors was gathered from farm owners/farm managers/veterinarian in a face to face interview.

Sero-positivity of BVDV

The samples were transported in ice box to University Diagnostic Lab (UDL), UVAS, Lahore and processed through antibody captured ELISA (IDEXX, Switzerland) as per manufacturer's recommendations to estimate the anti BVDV antibodies (Hanon *et al*., 2017). Serum samples having S/P values ≥ 0.30 (30%), S/P< 0.20 (20%) and 0.2 ≤ S/P<0.3 (20% to 29%) were considered as positive, negative and suspected, respectively.

Vaccine preparation

Bovine viral diarrhea virus characterized field isolate (BVDV 1/Bovine/Pak/UDL/018 5' UTR) was propagated on BVDV free Madin Darby Bovine Kidney (MDBK) cell line. $TCID_{50}$ was calculated (Reed and Muench, 1938) and viral fluid with $10^{6.9}$/0.1ml $TCID_{50}$ was inactivated using 0.01M binary-ethyleneimine (BEI) at 37°C for 24 h (Elhelw *et al*., 2022) following neutralization by sodium thiosulphate (10%). A vaccine dose of 2ml was prepared with the addition of 0.9 ml PBS with 0.1 ml inactivated virus fluid and 1ml montanide oil. Thiomersal sodium was added as a vaccine preservative at final concentration

of 0.01% (Fernandez *et al*., 2009). Finally, sterility of prepared vaccine suspension was ensured as recommended by (OIE, 2019).

Evaluation of BVDV vaccine

To evaluate and compare the humoral immune response induced by both self-prepared and commercial BVDV vaccine (Bovilis®, MSD), a total of 15 healthy cattle calves (BVDV Ag and Ab free) of ~6 months were divided into 3 groups (A, B, C) comprising 5 animals each.

Calves of group A and group B were primed with self-prepared and commercial vaccine respectively on day 0 and boosted on day 30 post-priming (S/C, 2ml). Animals of group C were kept as control (unvaccinated). Each group was kept at separate animals house. Control group also monitored for field virus infection. Clinical health status of each animal was observed at regular intervals during the experiment. Blood samples collected after every 2 months post vaccination (PV) (2-12 months) were tested by Ab capture ELISA kit (BVDV total Ab test, IDEXX, Switzerland).

Statistical analysis

Risk factors and vaccines s/p values were statistically analyzed through chi-square test and one way ANOVA in SPSS *v* 16.0.

RESULTS

Of the tested samples (n=1000) originated from five agro-climatic zones, overall 237 samples were detected positive (S/P ≥ 0.30) for anti-BVDV antibodies (Table I). Among the zones, highest sero-positivity was evidenced in low-intensity 34% (68/200) zone followed by rice wheat 27% (54/200), cotton-wheat 21% (42/200), mixed-crop19% (38/200) and rain-fed 17.5% (35/200) zones. Within rice-wheat zone, Kasur district was found with the highest sero-positivity 40% (20/50) followed by Narowal 32% (16/50), Sheikhupura 22% (11/50) and Hafizabad 14% (7/50) whereas in the mixed-crop zone, maximum sero-positivity was recorded in Jhang 42% (21/50) as compared to Sargodha 18% (9/50), Khushab 12% (6/50) and T.T. Singh 4% (2/50). In cotton-wheat zone, Bahawalpur district displayed more sero-positivity 32% (16/50), while other districts evinced 26% (13/50), 20% (10/50) and 8% (4/50) positivity in Pakpatan, Rahim Yar Khan and Lodhran respectively. From low-intensity zone, Muzaffargarh district has highest sero-positivity 56% (28/50) followed by D.G Khan 32% (16/50), Bhakkar 30% (15/50) and Mianwali 18% (9/50). Among the districts of rain-fed zone, Attock was found with highest sero-positivity 28% (14/50) while lowest in Rawalpindi 6% (3/50) (Fig. 1).

Table I. Sero-positivity of BVDV in five Agro-climatic zones of Punjab, Pakistan.

Agro climatic zones	Districts	No. herds	No. tested	No. positive	% Positive
Rice-wheat	Kasur	5	50	20	40%
	Sheikhupura	5	50	11	22%
	Hafizabad	5	50	7	14%
	Narowal	5	50	16	32%
	Sub-Total	20	200	54	27%
Mixed crop	Jhang	5	50	21	42%
	Sargodha	5	50	9	18%
	Khushab	5	50	6	12%
	T T Singh	5	50	2	4%
	Sub-Total	20	200	38	19%
Cotton wheat	Bahawalpur	5	50	16	32%
	Rahim Yar Khan	5	50	10	20%
	Pakpatan	5	50	13	26%
	Lodhran	5	50	4	8%
	Sub-Total	20	200	42	21%
Low intensity	M. Garh	5	50	28	56%
	D.G Khan	5	50	16	32%
	Mianwali	5	50	9	18%
	Bhakkar	5	50	15	30%
	Sub-Total	20	200	68	34%
Rain-fed (barani)	Attock	5	50	14	28%
	Chakwal	5	50	5	10%
	Jhelum	5	50	13	26%
	Rawalpindi	5	50	3	6%
	Sub-Total	20	200	35	17.5%
Grand total		100	1000	237	23.7%

Risk factors to BVDV

Among the risk factors, quarantine practice, housing management, abortion history and parity status showed significant association ($p<0.05$) whereas breeding system practices (AI/Natural) have no association ($p>0.05$) with occurrence of BVDV (Table II).

Comparison of humoral immune response induced by vaccines

The self-prepared vaccine was found to be free from bacterial, fungal and mycoplasma contamination. On 0-day PV, all the experimental animals were tested negative for

Table II. Risk factors association with the occurrence of BVDV.

Risk factors		No. tested	No. positive (%)	P value
Quarantine practices	Yes	446	52 (11.6%)	0.000
	No	554	181(32.6%)	
Housing management	Intensive	380	70(18.4%)	0.012
	Extensive	620	157(25.3%)	
Breeding system	AI	523	115(21.9%)	0.183
	Natural	477	122(25.5%)	
Abortion history	Yes	212	137(64.6%)	0.000
	No	788	85(10.7%)	
Parity status	≤ 2	550	40(7.2%)	0.000
	≥2	450	197(43.7%)	

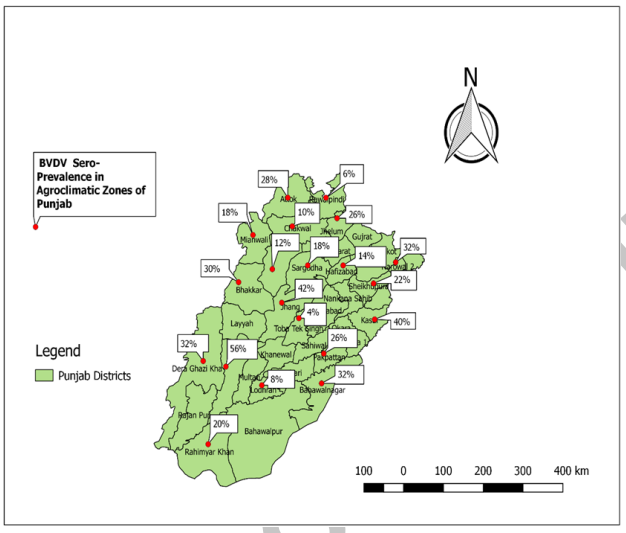

Fig. 1. Sero-epidemiology of BVD virus in agro-climatic zones of the Punjab through QGIS *v* 2.18.

BVDV Ab and Ag. The animals were injected with second shot of vaccines on 30th day post priming. On 2 months, a few experimental animals of both groups showed seroconversion however antibody titers were found non-protective (S/P<45%). In vaccinated groups, only 3/5 animal of group A and 4/5 of group B showed 31% and 35% S/P mean value respectively. Two animals of group A and one from B were suggested as doubtful for BVDV sero-positivity (≤0.2 S/P <0.3). In this study, protective antibody titers induced in imported vaccinated group B were found higher (46%) than self-prepared vaccinated group A (45%) on 4 month PV. Furthermore, on subsequent testing from 4th to 10th months protective antibody titer persisted in both vaccinated groups. Peak antibody titers (S/P) ranging from 57%-63% were observed on 6 month PV in both groups followed by decline on 8 and 10 months. The titers became non-protective on 12 month PV in both the vaccinated groups. Statistically both vaccines proved equally effective for their potential to induce humoral immunity (P > 0.05) however both vaccines produced significantly higher antibody titer (p<0.05) when compared with control group C throughout the experimental trial (Table III).

DISCUSSION

Recent expansion of intensive dairy farming in Pakistan has likely increased issues of animal health especially reproductive problems. Mostly asymptomatic reproductive pathogens pose serious challenges to dairy industry settings. Veterinary clinicians, most of the time link these pathogenic reproductive disorders with brucellosis, trichmoniasis while the potential role of BVDV causing abortion remains undiagnosed. Bovine viral diarrhea (BVD) is a disease that affects cattle and other ruminants throughout the world causing significant economic losses. The broad nature of the disease, its transmission and lack of treatment has made it globally enzootic and one of the most significant cattle diseases. Given the importance of BVDV, limited research data is available about prevalence and risk factors in southern Punjab of Pakistan (Raheem *et al*., 2020). Therefore, in order to find the true picture of BVDV this study was designed. In the present study, a cross sectional survey was carried out comprising of 1000 samples from 100 dairy herds of 5 agro zones of Punjab. In our hands, 23.7% overall animal level seropositivity of BVDV was observed that is relatively higher to the previous reported prevalence from southern (18.77%) Punjab of Pakistan (Raheem *et al*., 2020). This indicates increasing spread of BVDV in the country that might be due to insufficient control measures adoption by farmers. Comparative to our study, high prevalence (26.84%) of BVDV documented in a research study conducted in Ethiopia (Demil *et al*., 2021). Contrary to the present study, higher prevalence 32.9% and 42.5% has also been reported by different researchers in European countries (Asmare *et al*., 2018; González-Bautista *et al*., 2021). This disagreement in seropositivity among BVDV studies may be associated with the likely variation in herd size, farm management practices, housing systems and presence of PI animals which play an important role in the transmission of bovine viral diarrhea virus (Ortega *et al*., 2020). Moreover, in present study sero epidemiology observed among the five agro climatic zones with higher positivity rate in low intensity 34% zone followed by rice wheat 27%, cotton-wheat 21%, Mixed-crop 19% and Rain-fed 17.5% zones. Previously, variations in BVDV prevalence

in different geographical locations was reported in North Eastern India that is in accordance to our results (Singh *et al*., 2017). In addition to that research studies conducted in USA and Poland has also described difference in BVDV prevalence rate in multiple regions of the countries (Rypuła *et al*., 2020; Silveira *et al*., 2018). This regional variation could be associated with difference in epidemiological determinants that includes livestock population density, frequency of animal movement/animal trade, production system and risk factors (Krametter-Frötscher *et al*., 2007; Santman-Berends *et al*., 2017). Globally many risk factors have been found associated with the occurrence of BVDV. In our study higher prevalence was observed in herds where quarantine practices were not followed which is in line with the previous finding (Segura-Correa *et al*., 2016) and this could be due to the introduction of newly purchased animal in the herds with unknown BVDV status. The newly purchased animals might not have been screened for the presence of BVDV before introducing in the herd. Generally, animal purchasing influence the occurrence of infectious diseases, and in case of BVDV, the introduction of PI animals which play key role to spread the virus and maintain infection in cattle herds (Garoussi *et al*., 2019). This study showed that the BVDV higher seroprevalence in cattle with history of abortion than those without abortion history. This result coincides with previously reported study (Thapa *et al*., 2019) where significant association was found between BVDV sero-positivity and abortion. The higher chances of BVDV infection with the history of abortion indicates that the animals previously exposed to BVD virus. Mostly farmers tend to relate reproductive problems with other casual agents and did not conduct screening test to diagnose the true status of abortions in cattle. A greater prevalence was found in animals bred with natural mating (25.5%) compared to artificial insemination (21.9%). This finding is also in agreement with the research study (Fernandes *et al*., 2016) in Brazil that focused on the use of artificial insemination in animals and no association was found between breeding system and BVDV occurrence. Usually on farms bull service is used for the breeding purpose which is involved to disseminate the virus through semen, therefore seropositivity gets higher in animals bred with natural mating. Higher rate of BVDV infection was observed with the parity of cows. Findings of our study are in accordance to the previous study (Tesfaye *et al*., 2021) reported significant association between seroprevalence and the number of parity of cows. Age and parity are directly proportional to each other hence more prevalence in the animals with parity greater than 2 is likely due to the longer exposure time with BVDV pathogen. Regarding housing management, significant association was found with

Table III. Comparison of humoral immune response measured through ELISA post BVDV vaccination in cattle.

Day	SP values post BVDV vaccination																							
	0 (n=5)			2nd m (n=5)			4th m (n=5)			6th m (n=5)			8th m (n=5)			10th m (n=5)			12th m (n=5)		
Vaccine	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Animals 1	0.003	0.016	0.012	0.389	0.385	0.005	0.43	0.488	0.021	0.523	0.671	0.018	0.491	0.628	0.021	0.462	0.591	0.028	0.417	0.411	0.002
2	0.027	0.031	0.009	0.311	0.285	0.041	0.452	0.456	0.045	0.611	0.623	0.032	0.511	0.582	0.015	0.518	0.531	0.041	0.362	0.312	0.091
3	0.045	0.029	0.023	0.198	0.363	0.028	0.44	0.472	0.018	0.598	0.641	0.051	0.489	0.551	0.009	0.453	0.574	0.005	0.371	0.436	0.004
4	0.019	0.061	0.015	0.292	0.426	0.037	0.46	0.456	0.011	0.546	0.631	0.005	0.502	0.612	0.024	0.448	0.544	0.017	0.394	0.396	0.037
5	0.055	0.033	0.042	0.368	0.312	0.009	0.472	0.461	0.015	0.573	0.624	0.003	0.496	0.593	0.042	0.471	0.524	0.029	0.312	0.402	0.021
Mean	0.029	0.034	0.020	0.311	0.354	0.024	0.450	0.466	0.022	0.570	0.638	0.024	0.497	0.593	0.022	0.470	0.553	0.024	0.317	0.391	0.031

A, self prepared vaccine; B, commercial vaccine; C, unvaccinated (control).

the occurrence of BVDV. In our study, cattle housed in extensive system depicted higher serological positivity (25.3%) as compared to cattle housed in intensive system (18.4%). The results are in agreement with another study (Fernandes *et al*., 2016). Higher rate of seropositive animals in extensive housing system could account as the result of frequent contact of animals and the potential role of flies in transmittance of the virus from the PI animals as reported (Carlson *et al*., 2020).

Various approaches may be adopted to control the BVDV infection, involving the use of diagnostic assay to identify and culling of PI animals, enactment of biosecurity measures and the implementation of appropriate vaccination program. Vaccination is one of the main strategies to prevent the risk of fetal infection, abortion and the generation of PI animals. The major obstacles to follow BVDV vaccination program is cost-effectiveness of vaccines available in Pakistan. In this study, comparative antibody response was measured in calves elicited by commercial and self-prepared vaccine. Self-made vaccine and commercial Vaccine (Bovilis®, MSD) were administered twice (0, 30 day) to A and B group of five seronegative calves each and C group kept as control. In our hands, anti-BVDV-ELISA antibody titer were observed in both groups A and B two months post vaccination (pv), however antibody titers were non-protective (<45%). These findings are not in concordance with the findings of (Graham *et al*., 2003) who reported that strong antibody titers are produced in animals after two months pv. This disagreement may be due to variation in breed type, age, change in nutritional values of the fodder and environmental factors. In this study, protective antibody titer (>45%) were found on 4 month in vaccinated animals of both groups A and B. Similar findings were observed in previous study (Klimowicz-Bodys *et al*., 2021) who reported protective antibody titer induced in animals starting from 3 month to 4 month pv. Contrary to our findings, non-protective antibody titers were observed on 112 day in vaccinated animals using four types of ELISA (Álvarez *et al*., 2012). This disagreement may be due to the variation in the housing conditions, breed, age and different diagnostic kits. On 5-month post booster, maximum antibody titer (mean S/P 57%-62%) was recorded in vaccinated group and thereafter titer started to decline till 12-month. Surprisingly, antibody titer measured on 6 months was significantly higher than that in 2-month pv. These findings are in line with earlier study results conducted in Netherland where higher antibody response was measured in vaccinated animals after the 5 month of booster dose (Álvarez *et al*., 2012). Our study results are similar to another study (Patel *et al*., 2002) who reported antibody titer remained moderate to high 6 months post vaccination. This increasing trend in antibody response in vaccinated calves may be due to booster dose administration. Moreover, it was interesting to note that statistically the difference between antibody response induced by self-prepared and commercial vaccine was non-significant ($p>0.05$) throughout the experimental trial however antibody titer was significantly higher ($p<0.05$) when compared with control group C. Based on this study findings, it was noted that vaccines either commercial or self-prepared were capable to induce protective immunity that persisted for 4 to 10 month post vaccination.

CONCLUSIONS

BVD virus is circulating in cattle herds of various areas of each of the five agro-climatic zones of Punjab, Pakistan therefore animal producers may be advised to vaccinate their animals against BVD virus to combat silent killer of dairy industry.

ACKNOWLEDGEMENT

The authors are thankful to Livestock and Dairy Development Department, Punjab for aiding during sample collection. We would also like to thank Prof. Dr. Aamir Ghafoor and Dr. Rao Muhammad Ramiz for technical support.

Funding

The research work was funded by the Higher Education Commission (HEC), Pakistan research project # 4836 under NRPU.

IRB approval ethical statement

Current research work was undertaken with the institutional guide lines of Ethical Review Committee under DR/532. Samples were collected aseptically by veterinarian. No extra harm and procedure was done to animals throughout the study. Experiment was approved by ethical review board as described in IRB.

Statement of conflict of interest

The authors have declared no conflict of interest.

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