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Paper

Investigation of Peste des petits ruminants virus circulation in Uttarakhand, India: a step towards global eradication of PPR by 2030

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Abstract

This study was conducted to estimate the seroprevalence of Peste des petits ruminants virus (PPRV) and to determine the virus distribution in unvaccinated goats in the Pantnagar region of Uttarakhand state, India. A total of 212 serum samples from goats were collected randomly from various villages in three districts (Udhamsingh Nagar, Nainital, and Almora) of Uttarakhand. Serum samples were tested for anti-PPRV antibodies by a commercially available kit. RNA was extracted from the clinical samples and it was subjected to one-step RT-PCR, followed by virus isolation from positive samples. A total of 41 animals from various villages were found to be seropositive with a prevalence rate of 19.33%. PPR outbreaks were also reported from the Tarai region of Uttarakhand, and detection by PCR confirmed PPRV in 8 goats. Two representative swab samples were subjected to virus isolation in Vero cells and both samples showed typical cytopathic effects. The present study shows that PPRV is circulating in the Tarai region of Uttarakhand and mass vaccination for PPR must be followed in this region to increase herd immunity to a protective level. To the best of our knowledge, this is the first investigation of PPRV seroprevalence in unvaccinated goats of Uttarakhand, India.

Keywords

Global eradication, PPR-GCES, PPR-CP, Prevalence, Seroprevalence, Peste des petits ruminants virus

Introduction

Peste des petits ruminants (PPR) is caused by *Peste des petits ruminants virus* (PPRV) belonging to the genus *Morbillivirus* of the family *Paramyxoviridae,* within the order *Mononegavirales* (Amarasinghe *et al*., 2019). PPR mostly affects goats and sheep, but it also affects wild ruminants, pigs, and camels; cattle and buffaloes are asymptomatically infected with seroconversion, but other wild ruminants, pigs, and camels may show clinical signs and mortality (Albina *et al*., 2013; Rahman *et al*., 2016; Schulz *et al*., 2018). Goats are more susceptible than sheep to high mortality (Hussain *et al*., 2003). The annual global economic losses due to PPR are estimated between USD 1.4 to 2.1 billion (OIE, 2021) while losses in India are upto USD 2 million to USD 18 million which may go upto USD 1.5 billion (Bardhan *et al*., 2017) owing to morbidity, mortality, and productivity losses with trade limitations (Balamurugan *et al*., 2014). Sheep and goats in endemic areas may gain lifetime immunity after a natural infection, but naive animals may enable the virus to circulate continuously which may subsequently lead to an endemic state of the disease (Jones *et al*., 2016). Due to its trade restrictions and transboundary spread, PPR has a negative impact on the export of livestock and their products to countries where the disease is not present.

The state of Uttarakhand, India, comprises the Kumaon and Garhwal regions, which represent the Indian part of Central Himalayan flora. About 92% of the state is covered by mountains and hills, while 8% are Tarai plains. The Tarai region is a waterlogged alluvial plain with a gentle southeast slope, and deep and fertile, moist loamy soil, forming marshy land free from boulders and gravels. The state of Uttarakhand is divided into three geographical regions: 1, Upper hills which comprise of Uttarkashi, Chamoli, Rudraprayag, Pithoragarh, and Bageshwar; 2, Middle

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hills comprising of Tehri-Garhwal, Garhwal, Almora, and Champawat, the hill regions of Nainital and Chakrata tehsil of Dehradun; 3, Foothills which includes the remaining area of Dehradun and Nainital, Haridwar and Udham Singh Nagar. Udham Singh Nagar region of Uttarakhand lies in the Tarai region.

The total livestock population in the state is 4.42 million, out of which goats and sheep are 1.37 million and 0.28 million, respectively (https://ahd.uk.gov.in/pages/display/107-livestock-demography). About 94% of the goat population and 85% of the sheep population are reared by the poor people in Uttarakhand for meat purposes, although in rural regions, a large number of animals, mostly male goats are raised for religious sacrifices. Hence, goats are an important part of the livelihood of poor people in Uttarakhand.

PPR directly impacts the livelihood of poor farmers, so it needs to be eradicated globally. To reduce the PPR outbreaks in various states of India, the Government of India launched PPR-Control Programme (PPR-CP) in 2010 in the 11th five-year plan (2007-2012) intending to control and eradicate the disease in India (http://www.dahd.nic.in). This goal can be achieved by developing comprehensive active intensive surveillance programmes in enzootic areas, followed by intensive vaccination of all susceptible sheep and goats and their three subsequent generations. WOAH and FAO launched PPR-GEP in October 2016 for the worldwide eradication of PPR by 2030. This was followed by the launch of PPR- Global Control and Eradication Strategy (PPR-GCES) from 2017-2021 to be implemented in line with PPR-GEP (OIE and FAO, 2015). Therefore, to strengthen the PPR-CP and PPR-GCES, it is imperative to study the seroprevalence of PPRV from different regions of Uttarakhand state and isolation and identification of PPRV from clinical samples from the state.

This study aimed to generate epidemiological data related to seroprevalence and circulation of PPRV in the Tarai region of Uttarakhand. To the best of our knowledge, this is the first investigation of PPRV seroprevalence in the unvaccinated goats of Uttarakhand, India.

Materials and Methods

Serum sample size for seroprevalence study

The serum sample size for the seroprevalence study was calculated using the Sample Size Calculator in the Creative Research System software (http://www.surveysystem.com/sscalc.htm). Considering the 29% prevalence (Balamurugan *et al*., 2020) in Uttarakhand with a 95% confidence interval and margin of error of 4%, the sample size was determined for the estimation of seroprevalence. ArcGIS software was used to map the study area for estimating seroprevalence.

Serum samples from unvaccinated goats were randomly collected from October 2020 to September 2021 from the surrounding regions of Pantnagar, Uttarakhand which included villages of Udham Singh Nagar, Nainital, and Almora districts (Figure 1). A total of 212 serum samples from unvaccinated goats were collected. Out of total 212 serum samples, 150 serum samples were collected from the Udham Singh Nagar and Nainital districts (surrounding the Tarai region of Pantnagar), and 62 serum samples were from the hilly regions of the Almora district. The district-wise detail of sample collection is presented in Figure 1.

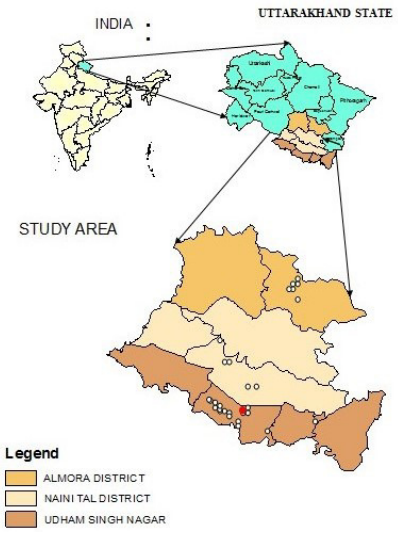

Figure 1 Arc GIS map of the study area used for seroprevalence study of PPR in the surrounding regions of Pantnagar. The three districts from which serum samples were collected are shown in the above map. The white dots in the map depicts the villages from which which serum samples

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have been collected. Pantnagar is depicted as red colour dot.

The samples were collected irrespective of the breed, and the sex of the animal. The samples were labeled with specific numbers for the identification of each animal. Briefly, 3 ml of blood from the jugular vein was collected in a vacutainer tube for the separation of serum. Serum was transferred into cryovials (Tarson Products Ltd, India) and stored at -20^0 C until competitive ELISA was conducted. The details of serum samples collected are given in Table I.

S. No.	District	Age						Total
		0-4 months	4-6 months	6-12 months	1 -2 years	2-4 years	4-6 years	
1.	Udham Singh Nagar	2	9	22	66	19	1	119
2.	Nainital	0	0	8	15	8	0	31
	Total	**2**	**9**	**30**	**81**	**27**	**1**	**150**
3	Almora	2	6	9	17	13	16	62

Table I Serum samples collected from unvaccinated goats of the surrounding regions of Pantnagar (Udham Singh Nagar and Nainital district).

Competitive ELISA for detection of anti-PPRV antibodies

Serum samples were tested by c-ELISA kit (ID Screen® PPR Competition, IDvet, France) for detection of anti-PPRV antibodies in terms of competition percentage (S/N %). The test was performed according to the manufacturer's guidelines.

The results were expressed as competition percentage (S/N %) which was calculated as follows:

S/N% = OD sample/ OD negative control x 100

The results were interpreted as follows:

Positive: S/N % ≤ 50 %

Doubtful: S/N % >50 % or ≤ 60%

Negative: S/N % > 60 %

Samples categorized as doubtful were retested by the same c-ELISA kit.

Clinical samples for PPRV isolation and identification

Clinical samples were collected from 19 goats, out of which 14 were suspected of PPRV infection and 5 apparently healthy, in the surrounding regions of Pantnagar from both Udham Singh Nagar and Nainital districts. Suspected goats were identified based on clinical signs like nasal discharge, sticky eyes, fetid diarrhea, high body temperature (105 ^{0}F), and mass mortality of in-contact goats (Figure 2). EDTA blood samples (Levram life sciences, India) and swabs (nasal, oral and rectal) were collected from the clinical outbreaks for virus identification and isolation. Each sample was labeled with a unique identifier with a specific number and date.

The details of clinical samples collected from goats have been given in Supplementary Table 1. A total of 37 clinical samples (blood, nasal, oral, and rectal swabs) were collected from 19 goats. Out of these nineteen goats, five were healthy, two were off-fed, and the remaining twelve goats showed clinical signs like nasal discharge, diarrhea, mouth ulcers, lachrymation, and dullness. Based on the clinical signs observed, clinical score has been depicted for each animal. Each clinical score has been given one score indicated by the "+" sign (Supplementary table 1). Apart from clinically suspected goats, samples were also collected from apparently healthy goats who were living in close contact with clinically suspected goats, but they did not show clinical signs of PPR. These healthy animals were given zero score as they didn't show any clinical signs.

The samples were immediately put inside an ice box and transferred to the lab. The clinical samples were stored at -40^0 C until the test was performed. Serum samples for testing anti-PPRV antibodies by c-ELISA were also collected from these suspected cases.

Swabs were immersed in 3 ml of phosphate-buffered saline (PBS) (pH 7.4) and homogenated by gentle swirling and squeezing. The homogenate was then filtered through a 0.22 µm Millipore syringe filter under aseptic conditions. The filtrate was used for RNA extraction and as inoculum for virus isolation.

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Figure 2 Clinical signs of PPR in goats tested in this study: (a) ulceration on mouth; (b) nasal discharge; (c) diarrhoea; (d) lachrymation.

Identification of PPRV from clinical outbreaks

The RNA was extracted from clinical samples using TRIzol-S Reagent (SRL, India), following the protocol described by Chomczynski and Sacchi., 1987 (Chomczynski and Sacchi, 1987) with slight modification. The extracted RNA was stored at -20^0 C until further use. The purity of extracted RNA was determined based on A260: A280 ratio using Biospectrometer, Eppendorf.

The presence of PPRV nucleic acid in the clinical samples (whole blood, nasal swab, oral swab, and rectal swab) was detected by a one-step RT-PCR kit (Himedia Laboratories Private Limited, India). The PCR products were analyzed by agarose gel electrophoresis using a standard protocol.

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Isolation of PPRV from clinical samples and their characterization by SDS-PAGE analysis

Tested positive samples by the RT-PCR assay were subjected to virus isolation. The Vero cell line (ATCC,CCL-81) used for the isolation of PPRV from clinical samples was provided by Indian Veterinary Research Institute (IVRI), Mukteshwar, Uttarakhand. Vero cells were cultured in EMEM (Himedia Laboratories Private Limited, India) with 10% fetal bovine serum (Himedia Laboratories Private Limited, India) and antibiotics (streptomycin and penicillin). For virus isolation, 25 cm^2 tissue culture flasks with a confluent monolayer of Vero cells were taken. Media was decanted from the tissue culture flask (Greiner, Germany) and 1 ml filtered sample was inoculated on to confluent monolayer of Vero cells. Flask was then incubated at 37^0 C for one hour for the adsorption of the virus with gentle rotations every 15 minutes, finally, the inoculum was removed and 5.0 ml of EMEM medium supplemented with 2% FBS and antibiotics were added to the flask. The flask was then incubated at 37^0 C temperature. Vero cells were observed under an inverted microscope at 24 hrs, 48 hrs, 72 hrs, and 96 hrs to observe the cytopathic effect (CPE). After the appearance of 70-80 % CPE, the flask was freeze-thawed three times, and contents were transferred to a 50 ml centrifuge tube followed by centrifugation at 5000 rpm for 30 minutes at 4°C. The supernatant containing the virus was transferred to a separate collection tube and stored at -20°C till further use. The harvested virus was then confirmed by RT- PCR and SDS-PAGE. PPRV Sungri/96 vaccine strain (Raksha PPR® Indian Immunologicals Pvt. Limited) was used as a positive control in SDS-PAGE analysis.

Results

Seroprevalence of PPRV in the surrounding regions of Pantnagar

Out of 119 serum samples from nearby villages of Pantnagar, 35 samples tested positive for anti-PPRV antibodies with a prevalence rate of 29.41% (Table II). Out of 31 serum samples from the Nainital district, 4 samples tested positive for anti-PPRV antibodies with a prevalence rate of 12.90% (Table II). Out of 150 serum samples from the surrounding regions of Pantnagar, 39 samples tested positive with a prevalence rate of 26% (Table II). Out of sixty-two serum samples from the Almora district, two serum samples tested positive, and sixty serum samples were negative for anti-PPRV antibodies with a prevalence rate of 3.22 % in Almora district (Table II).

S. No.	Name of the district	No. of positive serum samples	No. of negative serum samples	Total serum samples tested	Seroprevalence
1.	Udham Singh Nagar	35	84	119	29.41%
2.	Nainital	4	27	31	12.90%
	Total	**39**	**111**	**150**	**26.00%**
3.	Almora	2	60	62	3.22%

Table II Seroprevalence in the surrounding regions of Pantnagar in Udham Singh Nagar district.

Out of 212 serum samples from Udham Singh Nagar, Nainital, and Almora districts, a total of 41 serum samples tested positive for PPRV antibodies with an overall prevalence rate of 19.33%.

Age-wise percentage seropositivity in the surrounding regions of Pantnagar (Udham Singh Nagar and Nainital district)

The percentage seropositivity of PPRV was estimated age-wise in the sampled animals. The animals were divided into six different groups (Supplementary Table 2). At 0-4 months of age, two serum samples tested positive. At 6-12 months of age, the seropositivity rate was 20.00%. At 4-6 months and 2-4 years of age, the seropositivity rate was 44.44%. At 1-2 years of age, 18.51% seropositivity rate was observed.

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Detection and prevalence of PPR virus

Extracted RNA was subjected to one-step RT-PCR. In the present study, 37 clinical samples were collected from 19 goats, out of which 8 animals tested positive. Hence, 42.10% of total tested animals turned positive for PPRV in the surrounding region of Pantnagar (Supplementary Table 1).

Out of eight animals found to be positive in RT-PCR, seven animals (G295, G296, G298, G299, G300, G301, G229) were found to be seropositive when tested with ID Screen® PPR Competition, France.

Isolation of PPRV

Samples positive by one-step RT-PCR were subjected to virus isolation in Vero cells. Two samples comprising one pooled and one rectal swab sample were inoculated to Vero cells. Swabs from both goats (Animal number G295 pooled swab and G296 nasal swab) showed typical cytopathic effect (CPE) of PPRV i.e. rounding and fusion of cells (Figure 3-1) after 48 hr. This was followed by a complete detachment of the cell monolayer in 48-72 hours in comparison to the vaccine strain Sungri/96 which showed complete CPE in 4-5 days without complete monolayer detachment (Figure 3-2), however, the cell control monolayer did not show any changes (Figure 3-3).

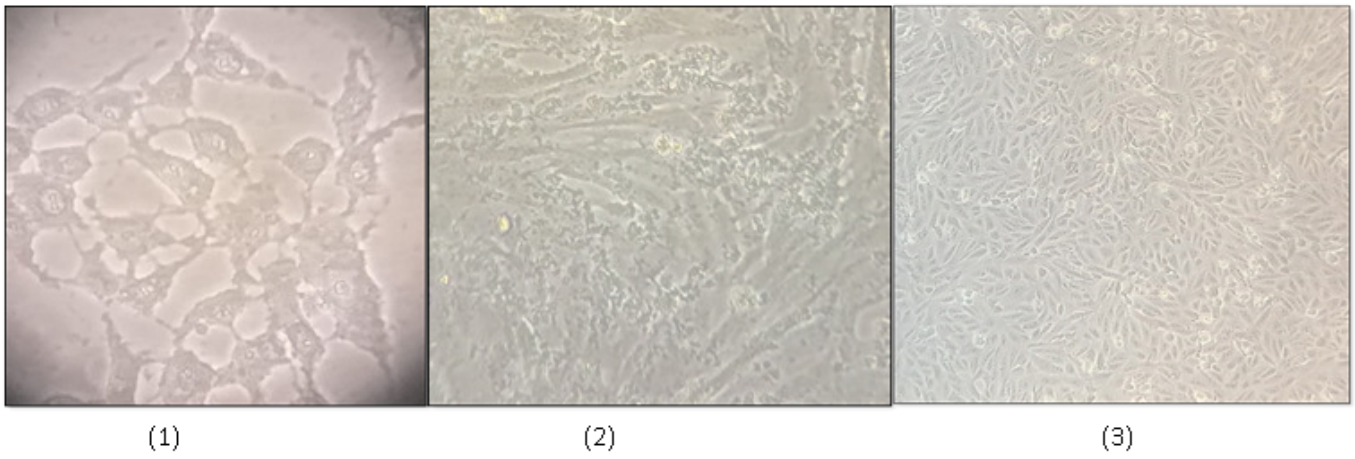

Figure 3 Cytopathic effect in field isolate, vaccine strain, and negative control wells: (1) isolation of PPRV in Vero cell culture (40X). PPRV field isolate, rounding and fusion of Vero cells (48-72 hours post-infection); (2) cytopathic effect induced by PPRV/Sungri/96 ; (3) Uninfected control Vero cells.

Confirmation of the isolated field PPRV

The presence of PPRV isolates and reference vaccine virus (Sungri /96) were confirmed by one-step RT-PCR and SDS-PAGE. Both isolates and the vaccine strain were found positive by M gene-specific one-step RT-PCR yielding an amplicon of 124 bp. In SDS- PAGE, PPRV isolates, and PPR vaccine strain revealed F1 protein (50 kDa), H protein (67 kDa), N protein (58 kDa), M protein (38 kDa), and P protein (86 kDa), which indicated that the isolated viruses were PPRV (Figure 4).

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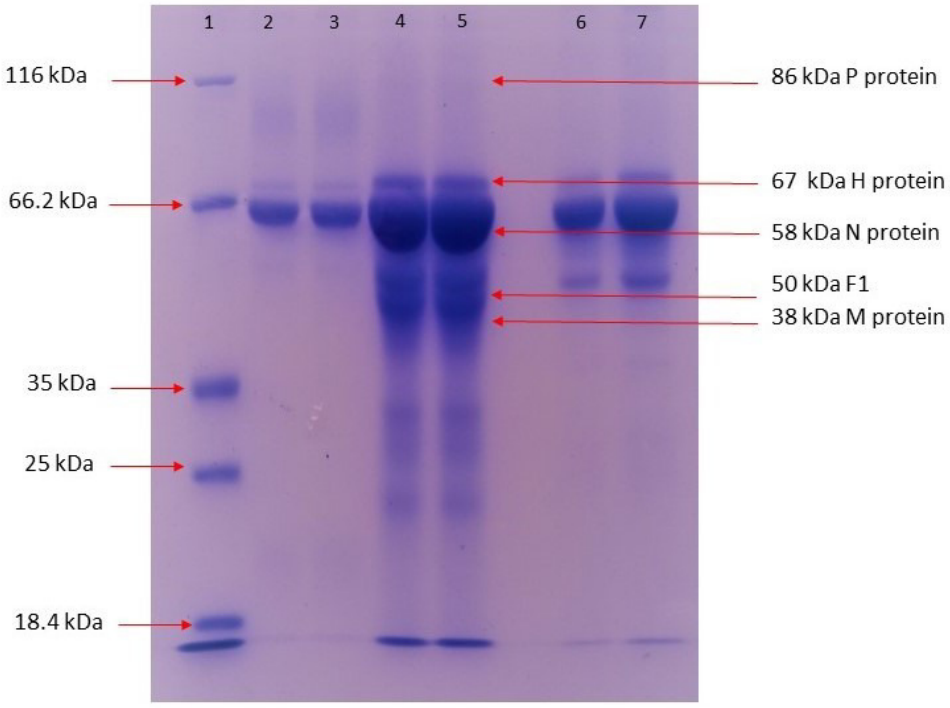

Figure 4 SDS- PAGE analysis of the PPRV field isolates and their characterization. Lane 1: Unstained protein marker (Thermo Fisher Scientific, USA), Lane 2-3: PPRV/Sungri/96, Lane 4-5: PPRV isolates, Lane 6-7: Cell culture PPRV- Sungri/96 vaccine.

Discussion

PPR occurs throughout Africa (apart from the most southern countries), the Middle East, Turkey, West and South Asia and China. Global eradication of PPR will directly benefit the livelihoods and stability of millions of pastoralists and livestock smallholders in affected countries.

Following the global eradication of Rinderpest, attention has turned to PPR with aims to eradicate by 2030 (OIE and FAO, 2015). In India, PPR is endemic. The government of India has initiated a PPR control program (PPR-CP) under which susceptible domestic animals more than 4 months of age are to be vaccinated. Some of the states in India practiced focused vaccination (vaccination limited to the place of the outbreak with a radius of 3-10 km to contain the disease spread) in the outbreak situation for the control of the PPR since 2002 (Singh *et al*., 2009). However, the strategic mass vaccination program (vaccination covering the entire small ruminants population above the age of 4 months old and subsequent biannual/annual vaccination of naïve young population and unvaccinated animals) was implemented in some of the states through the national control program on PPR (PPR-CP) since 2010 and 2014 for the control and eradication of the disease even before the global framework was planned . (Balamurugan *et al*., 2016) In India, several PPR outbreaks go unrecorded due to the under-reporting of clinical outbreaks by the goat owners.

In a recent study by Balamurugan and colleagues (Balamurugan et al., 2020), it was found that a few sporadic PPR outbreaks were reported in the states of Uttarakhand, Himachal Pradesh, and Jammu and Kashmir. Nevertheless, systematic epidemiological surveys for the state or region, or zone have not been conducted except for a few studies (Balamurugan *et al*., 2020, 2019, 2014). Understanding PPRV circulation in a given geographical region followed by implementing proper control measures is then paramount to eradicating PPR. In the Tarai region of Uttarakhand, so far, no epidemiological study on PPR has been conducted. Therefore, the present study was undertaken to estimate the seroprevalence and circulation of PPRV in and around the surrounding regions of Pantnagar.

In this study, an overall prevalence rate of 19.33% was recorded. However, a higher seroprevalence (29.35%) was reported by Balamurugan and colleagues (Balamurugan *et al*., 2020) in which the serum samples were randomly collected from sheep and goats of unknown antibody status. In our study, the serum sample has been taken only from the unvaccinated goats and is the genuine indicator of PPRV seroprevalence/ virus circulation in unvaccinated animals, irrespective of antibodies generated due to vaccination. Seroprevalence studies have also been done in other parts of India in which the seroprevalence ranged from 29% to 57% (Balamurugan *et al*., 2014; Balamurugan *et al*., 2019; Balamurugan *et al*., 2020; Chavan *et al*., 2009).

The seroprevalence of PPRV was also estimated age-wise in the sampled animals. The high prevalence in the 2-4

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years age group could be due to the frequent purchase/movement of the goats of this age group for trade purposes. In endemic and developing countries frequent animal movement is one of the prime reasons for disease spread (Chevalier *et al*., 2004). The prevalence of PPRV antibodies below one year of age could be because goats from 4 months to upto one year of age are highly susceptible to PPRV infection (Venkataramanan *et al*., 2005).

In goats, anti-PPRV maternal antibodies decline from the third month onwards and recede below the protective level by the fourth month of age. These maternal antibodies were detectable up to 6 months of age in goats (Balamurugan *et al*., 2012). Therefore, seropositivity of anti-PPRV antibodies in unvaccinated goats after 6 months indicates the PPR infection.

In the present study, clinical samples were collected from nineteen goats, out of which eight animals turned out positive, thus indicating circulation of PPRV in the Pantnagar region. This could be because Uttarakhand state unlike Haryana, Punjab, Uttar Pradesh, and Himachal Pradesh had not adopted a mass vaccination campaign in line with PPR- CP since 2014 (Balamurugan *et al*., 2020). To obtain the desired herd immunity to prevent active transmission of the virus, 80-90% vaccination coverage of the population at risk is required to control and eradicate PPR, taking into account other epidemiological parameters (Zahur *et al*., 2009). For that, vaccination covering the whole population initially, subsequently, bi-annual vaccination covering the naïve young population needs to be adopted, as per the PPR control and eradication strategic plan (Balamurugan *et al*., 2020). However, the difficulty in maintaining an effective cold chain in rural areas is the major constraint to achieving desired 80% herd immunity. Also, the small ruminant population exhibits rapid turnover due to births, purchases of animals, and deaths due to various causes (Yirga *et al*., 2020). Therefore, more frequent vaccination may be required to maintain herd immunity at sufficient level (Njeumi*et al*., 2020). Also, post-vaccination evaluation of herd immunity is essential as it enables evaluation of the immunogenicity of vaccines and efficiency of vaccine delivery (OIE and FAO, 2015).

The present study signifies that PPRV is in circulation in and around the Tarai regions of Uttarakhand. Therefore, regular mass vaccination of the flocks with sero-surveillance and evaluation of post-vaccination herd immunity is essential to control the PPRV.

The above study has generated epidemiological data related to the prevalence and seroprevalence of PPRV in the Tarai region of Uttarakhand which will contribute to the PPR-GCES and subsequently eradication of PPRV by 2030.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study does not include any animal experiment and no ethical approval is needed.

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