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# Identification and Phylogenetic Analysis of Peste des Petits Ruminants (PPR) Virus Isolates from India

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## ABSTRACT

In this study we performed the sequence and phylogenetic analysis of peste des petits ruminants (PPR) virus isolates recovered from natural disease outbreaks in goats of central India. A total of 58 clinical samples comprised of blood, nasal/oral/rectal swabs were collected from 3 natural PPR disease outbreaks. The samples were tested by using N protein based MAb based sandwich ELISA (sELISA) and subsequently RT-PCR targeting Fusion (F) and Nucleoprotein (N) genes of PPR virus. The F and N gene specific PCR amplicons were sequenced and assessed for sequence variations with existing and circulating PPR virus strains and vaccine isolates. The results of this study presents PPR virus antigen in 48.27% clinical samples (28/58) using sELISA confirming this outbreak of PPR in goats. Amplification with primers F1/F2 for F gene yielded an expected amplicon of 372 bp and N1/N2 primer sets yielded an amplicon of 463 bp for N gene in reference vaccine virus as well as 28 field samples. In phylogeny, different patterns of branching based on N and F gene sequences and clustering of many of the central Indian PPR virus isolates with isolates from bordering countries was seen. The results confirm that heterogeneous population of PPR virus isolates is circulating in India which moreover emphasize the significance of molecular methods to understand the epidemiology and diversity of PPR virus in the country. The information from such studies will help in achieving the target of controlling and eradicating the disease, especially when the country is planning to launch the control programs in many states after the successful stamping out of the Rinderpest.

**Key words:** Peste des petits ruminants, outbreak, epidemiology, goats, India, fusion gene, nucleoprotein gene, sequence analysis

## INTRODUCTION

Peste des petits ruminants (PPR) is an extremely acute, fast spreading, communicable transboundary viral disease of small ruminants leading to 100% morbidity and up to 90% mortality (Dhar *et al*., 2002). The PPR virus comes under genus *Morbillivirus*, family Paramyxoviridae, order Mononegavirale (Murphy*et al*., 1999). The PPRV infection has been documented in India and many adjoining Asian countries such as China, Bangladesh, Pakistan (Munir *et al*., 2012), Tajikistan, Iran, Sudan (Balamurugan *et al*., 2012). In India, the disease has been estimated to cause colossal economic loss of approximately up to Rs. 1.8 billion (US$ 39 million) every year (Singh *et al*., 2004).

Since, its first report from Tamil Nadu in 1989 by Shaila *et al.* (1989), the disease has acquired a prime status in India and spreading to many states of the country (Venkataramanan *et al.*, 2005; Balamurugan *et al.*, 2014). The PPRV is classified into 4 genetically distinctive lineages (1, 2, 3 and 4) based on sequence analysis of fusion protein (F) gene (Balamurugan *et al.*, 2014). This grouping of PPRV into lineages is of paramount importance in analyzing the molecular epidemiology and global spread of PPRV. However, due to unending upsurge in circulation of PPRV strains amongst the vulnerable population in endemic countries like India, it's needed to explore and understand the molecular niceties of the circulating PPRV strains. This study was initiated to detect and characterize the PPRV in outbreaks suspected for PPR among indigenous goats of Madhya Pradesh (a central part of India).

## MATERIALS AND METHODS

**Virus isolates:** The clinical samples viz. blood, nasal/oral/rectal swabs were taken from three outbreaks in goats that occurred at different locations (Katni, Balaghat and Hoshangabad) of Madhya Pradesh during 2007-2008. A total of 58 goats with symptoms suggestive of PPR infection were sampled during disease investigations. Initially, all the PPR suspected samples were tested by using N protein based MAb based sELISA kit, developed at IVRI Mukteswar following the procedure as described by Singh *et al.* (2004). The samples found positive in sELISA were further subjected to RT-PCR targeting N and F genes and one sample each from representing areas was subjected for sequence analysis. To optimize the PCR conditions, lyophilized PPR vaccine (Sungri 96 isolate) was procured from Indian Veterinary Research Institute, Mukteswar, India.

**Viral RNA isolation and quantitation:** The viral RNA extraction from the clinical samples collected (blood, nasal/oral/rectal swabs) was done using Spin viral RNA kit (Zymo Research, UK). In brief, for 200 μL of sample an amount of 600 μL viral RNA buffer was added, mixed and shifted to a Zymo-Spin IC TM Column in a collection tube and centrifuged at 10,000 rpm for 1 min. The flow was discarded and column was washed with 300 μL RNA wash buffer at 10,000 rpm for 1 min. Once more the flow was decanted and washing was repeated. The column was positioned into RNAse-free micro centrifuge tube with addition of 10 μL of DNAse/RNAse-free water which was allowed to stand at room temperature for 1 min, followed by centrifugation at 10,000 rpm for 1 min to elute RNA. The isolated RNA was analyzed in Nanodrop spectrophotometer (ND-1000, USA) and quality of RNA was adjudged on the basis of optical density ratio at 260: 280 nm.

**Reverse-transcription-PCR:** The RT-PCR conditions were optimized using the N gene and F gene specific primers as proposed by Forsyth and Barett (1995) and Kerur *et al.* (2008). A single step RT-PCR (BioRT, Taurus Scientific, USA) procedure was adopted for the first strand cDNA synthesis and subsequently PCR. The conditions of PCR as proposed by Kerur *et al.* (2008) were followed in a Thermocycler (GeneAmp®, PCR System 9700, Applied Biosystems, USA). The master mix and template were mixed together with N and F genes specific sense and anti-sense primers. Amplicons of expected size were visualized on 1% agarose gel as a specific single band in the gel documentation system (AlphaDigiDoc™ RT, UK). Consequently, PCR amplicons were visualized purified using NP Gel Extraction kit (Taurus Scientific, USA). Furthermore, the selected amplicons were subjected for sequencing using sense primer used in RT-PCR.

**Sequence comparison and phylodynamics:** The nucleotide sequences obtained during the study were submitted in the NCBI GenBank sequence database with accession numbers GQ122188, GQ122189, GQ122190 for N gene and FJ858770 and FJ858771 for F gene, respectively. The sequence chromatogram files were visualized using Chromas 2.33 version software. Mega 4 BLAST was performed with the deduced sequence within the non-redundant nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to approve the existence of PPR virus specific sequences. The sequence data retrieval of PPR virus specific sequences was done from public nucleotide database. Sequences were downloaded from various parts of India as well as neighbouring countries for percent nucleotide and amino acid identity analysis. The analysis was done using Neighbor-Joining method, maximum composite likelihood computation and 1000 bootstrap replicates (Saitou and Nei, 1987; Tamura *et al*., 2007).

## RESULTS AND DISCUSSION

Peste des petits ruminants (PPR) is an important transboundary calamity of small ruminants not only in India but globally, inflicting significant loss in terms of morbidity and mortality. The PPR disease has been identified a as one of the main impediments in the development of the small ruminant industry in India. The sheep and goats husbandry play an integral role in sustainable agriculture and employment in India with estimated population of 104.5 million goats and 71.6 million sheep as per 18th livestock census (2007) (http://dahd.nic.in/dahd/upload/BAHS_2010.pdf). This sector of animal husbandry is often being the responsibility of tribal, landless and marginal farmers. The first transboundary nature of one of the isolates of PPR virus from Gujarat region of India was reported by Wang *et al.* (2009), in which data suggestive of cross-border transmission of PPRV infection into Tibet was sighted. It was hypothesized that virus spread could be due to the western and south western Ngari topography, which might have allowed frenzied animal movement and trade between Tibet and bordering nations including India and Nepal. The expansion of diseases of such gravity from neighboring countries is a matter of major apprehension and challenge to the government agencies in India. Looking into the socio-economic importance of this disease of marginal farmers, PPR has been identified as one of the important priority diseases with a mandate to stamp it out after achieving the success for Rinderpest eradication in 2011.

In the present study, we investigated the cause of disease outbreaks in native goats of central India, where majority of the human population is rural (73.3%) and tribal (19.9%) and largely depends on goat husbandry for their livelihood. As per the latest Indian Government livestock census (19th livestock census of 2012), the state of Madhya Pradesh had 3.08 lakh sheep and 80.14 lakhs goats (http://dahd.nic.in/dahd/WriteReadData/Livestock.pdf). In spite of several control measures and regular vaccination of the susceptible population with PPRV vaccine, its outbreaks are still recorded in many parts of the state. Thus, to assess the similarity and divergence among the field PPR virus strains of the central part of India as well as compare their similarity to the hitherto isolates and vaccine strains, partial F gene sequences (372 bp) and N gene sequences (463 bp) were amplified, sequenced and investigated.

During the present study, PPR virus antigen was detected in 28 (48.27%) clinical samples out of 58 nasal/faecal samples from goats showing symptoms and lesions suggestive of PPR using sELISA. Amplification with primers F1/F2 for F gene yielded an expected amplicon of 372 bp and N1/N2 primer sets yielded an amplicon of 463 bp for N gene in reference vaccine virus as well as 28 field samples (Fig. 1). The remaining 30 samples taken from affected goats failed to produce the targeted amplification.

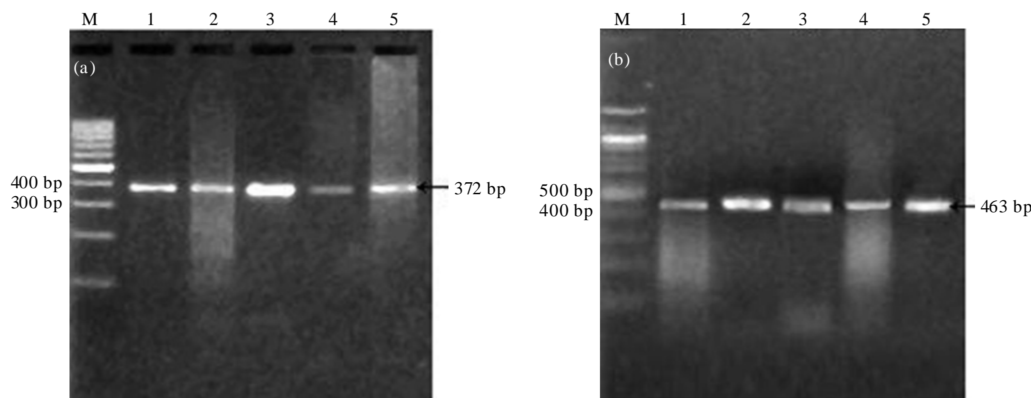


Fig. 1(a-b): RT-PCR amplification of PPR virus specific Fusion (F) and Nucleoprotein (N) genes, (a) Arrow indicates F gene specific a mplicon of 372 bp. M: 100 bp DNA ladder, 1: vaccine virus, 2-5: Field samples and (b) Arrow indicates N gene specific amplicon of 448 bp. M: 100 bp DNA ladder, 1-4: Field samples and 5: vaccine virus

Two of the representative RT-PCR positive samples of the F gene (FJ858770 and FJ858771) and three for N gene (GQ122188, GQ122189, GQ122190) were sequenced to inspect genetic similarity between PPR virus isolates. A sequence appraisal analysis of the F and N gene viral sequences revealed that the PPR viruses from distant regions showed more variations in their N gene sequences than in their F gene sequences. Hitherto studies have also proposed that PPRV N gene yield better picture of molecular epidemiology in comparison to F gene (Kerur *et al*., 2008).

Sequence analysis of the F gene of field PPR viruses (Ind MP Balaghat-08/FJ858770 and Ind MP Hosangabad-08/FJ858771) revealed that, the two isolates shared a homology of 95% while an earlier report from Madhya Pradesh showed 100% homology among the isolates recovered from outbreaks of PPR in 2004 (Balamurugan *et al*., 2010) and there were no differences with the Sungri-96, the vaccine strain. The isolates analyzed during the present study showed more than 95% homology at amino acid level with majority of the Indian and Asian isolates (Fig. 2). One of the isolate (Hosangabad-08/FJ858771) showed 98.2% homology with the vaccine virus sequence (Sungri-96) while another one (Balaghat-08/FJ858770) showed 95.5% homology. On comparing field isolates with vaccine strain, 8 changes in amino acid triplet positions were also noticed. Based on the translational data of partial F gene, changes were seen at positions viz. 1, 4, 5, 7, 10, 113-115, which could be responsible for alteration in the function of the protein.

The phylogenetic analysis results based on partial length F gene of 372 bp are presented in Fig. 2. For the analysis, 36 F gene sequences were retrieved from the GenBank database using MEGA 4. The analysis showed that the field isolates makes two different clusters. The Balaghat-8 isolate cluster with the PPR virus isolates from Gujarat, Turkey, Pakistan, Tamil Nadu, Madhya Pradesh (old isolates) and Utter Pradesh. However, another isolate (Hosangabad-08/FJ858771) clustered with isolates from China-Tibet isolates and one isolate from Gujarat (Bsk-Guj-05/DQ267186). The same isolate from Gujarat was earlier reported to be closely related to Tibetan isolate by Wang *et al.* (2009). All of these isolates belong to Asian lineage 4. The results suggest that PPR virus isolate (Hosangabad-08/FJ858771) closely related with Tibetan isolate is in circulation in the central part of India, which either has entered from neighboring state Gujarat or has Tibetan origin but needs further studies to confirm this.

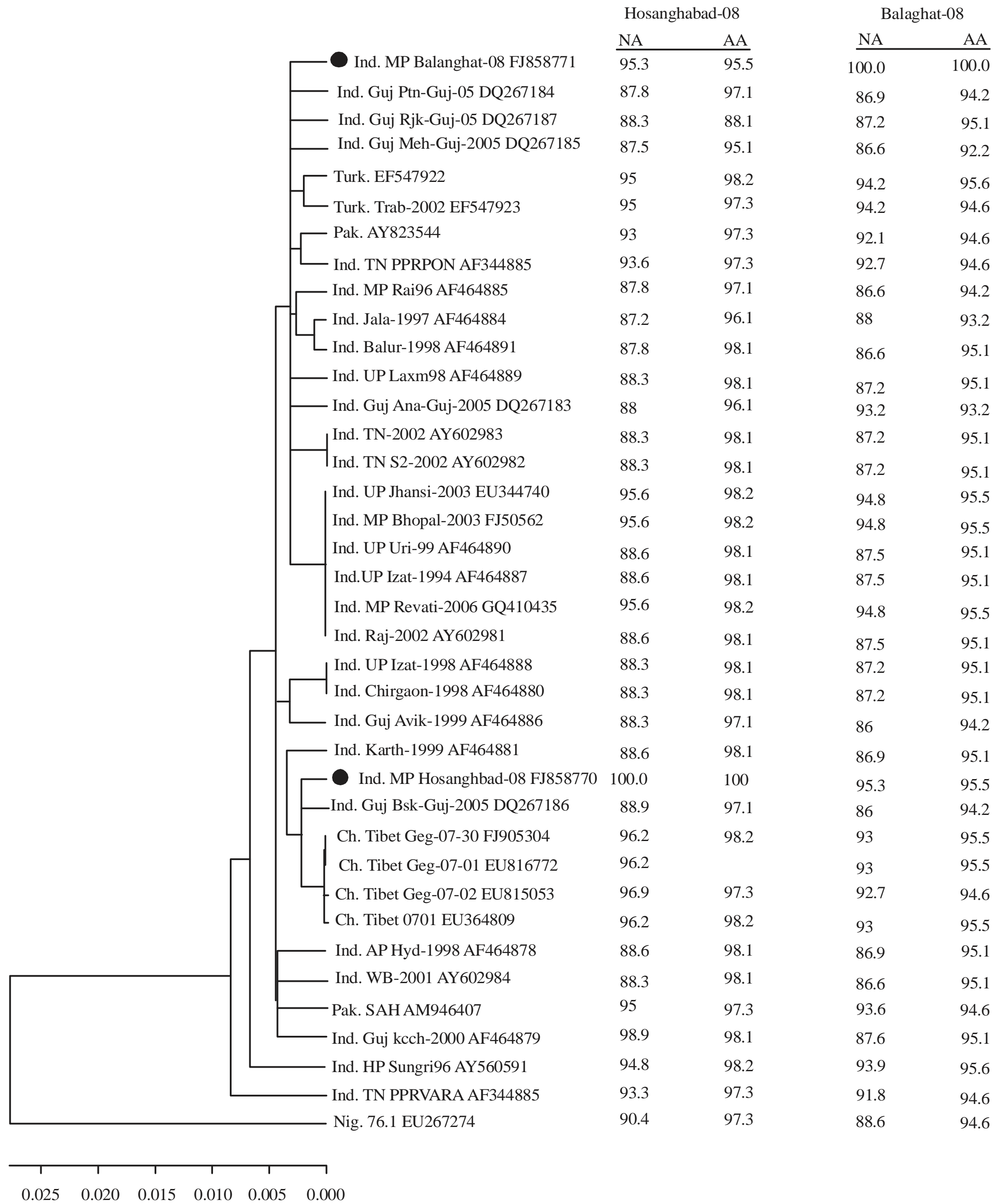


Fig. 2: Phylogenetic relationship of PPR virus of central India with other virus isolates based on the F-gene partial sequences. The central Indian isolate MP Balaghat clustered with all other indian isolates while another isolates MP Hoshangabad formed a separate cluster with China-Tibet strains and one Indian strain from Gujarat (Ind Guj Bsk-Guj-2005). Bar represents the genetic distance, i.e., number of substitutions per site

Analysis of 463 bp sequences of N gene of three PPRV isolates (Ind MP Balaghat-08/GQ122188, Ind MP Katni-08/GQ122189 and Ind MP Hoshangabad/GQ122190) have shown that the PPRV

isolates of present study as well as other Indian isolates have higher similarity of 95-99.2%. All the three field isolates showed 93-93.9% homology with the vaccine virus sequence (Sungri-96). The data on PPR virus from India confirms detection of virus of lineage IV. Higher homology of all the isolates clustered all three field isolates in a single cluster with majority of the PPR virus isolates from China-Tibet (Fig. 3). The amino acid triplet analysis revealed changes at 7, 34, 37, 50, 71, 80, 82, 89 and 125. At two positions (80 and 89) change in proline to leucine or serine was seen while comparing field isolates with vaccine strain (Sungri-96). The divergence from vaccine strain could

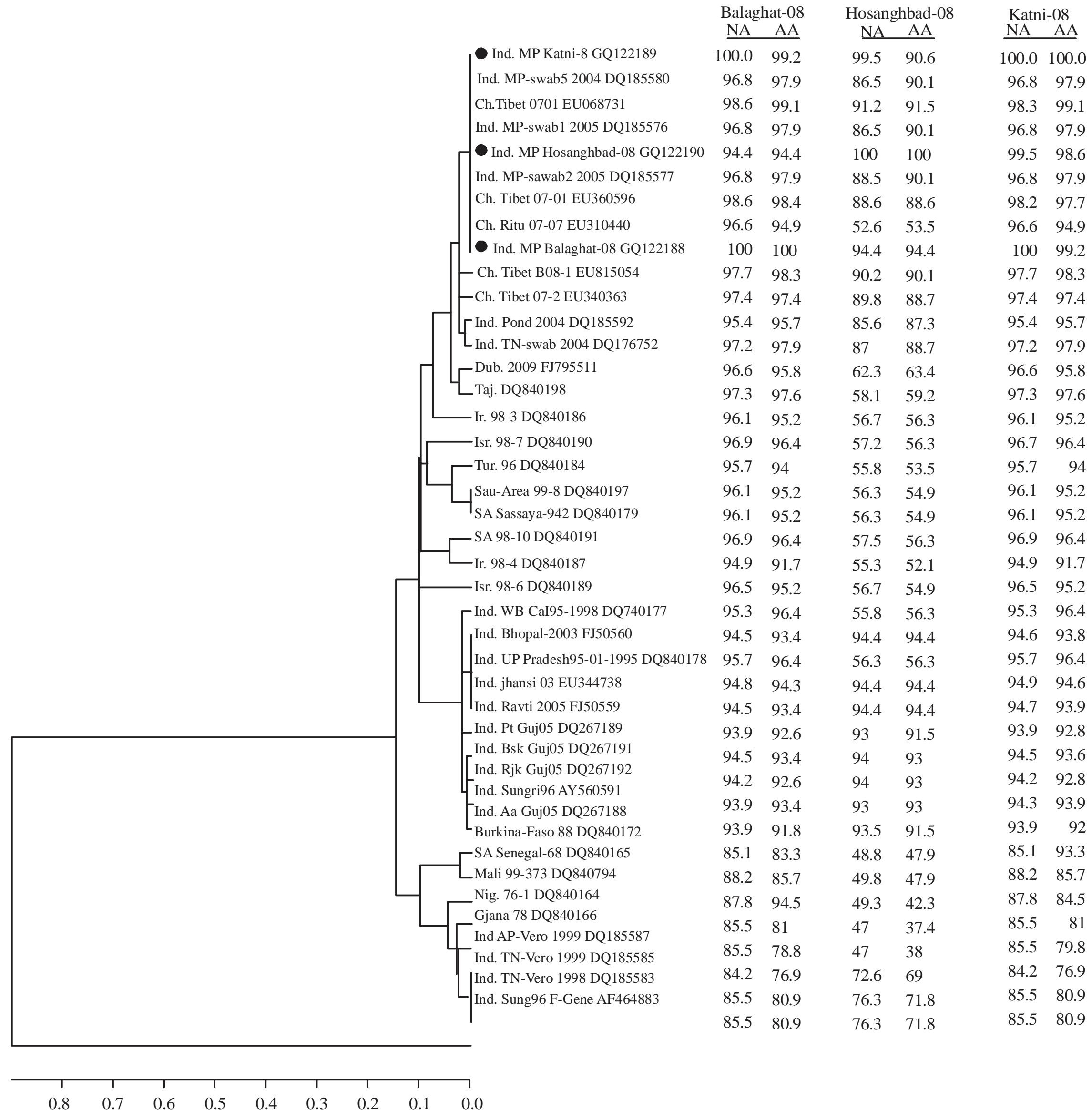


Fig. 3: Phylogenetic relationship of PPR virus of central India with other virus isolates, based on the N gene partial sequences. All the central Indian isolates clustered into a separate branch with China-Tibet strains with few other Indian isolates. Bar represents the genetic distance, i.e., number of substitutions per site

be the reason of number of outbreaks occurring in field in spite of vaccination or evolution of a new strain of the virus not protected/partially protected by available vaccine strain (Sungri-96) but need further studies to confirm this assumption. The findings highlight the potential for spread of these PPRV strains into areas that have never been documented previously. It is an apprehension that these PPRV isolates might be present across a greater area than presently assumed. It is possible that PPRV has spread into from many other bordering countries but needs more elaborative research on this issue.

## CONCLUSION

The study confirms detection and circulation of PPRV in indigenous goat population in India. The sequence and phylogenetic analysis confirms that the PPR virus that circulates in this region of the country is of the lineage IV and cluster closer with PPR viruses from China-Tibet. The unbarred animal's movement and their trade could be one of the accountable reasons for the transmission of this virus. The results emphasize the importance of molecular methods to understand the epidemiology and evolution of virus in the country on a larger scale. The information from such studies will help in achieving the target of controlling and eradicating the disease, especially when the country is planning to launch the control programs in many states after the successful stamping out of the Rinderpest.

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