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Research Article

STUDIES ON CO-INFECTION OF *PESTE DES PETITS RUMINANTS* VIRUS AND ORF VIRUS *IN VITRO*

P. Giridharan* and Y. Krishnamohan Reddy

Vaccine Research Centre–Viral Vaccines, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai-600051, Tamil Nadu, India

ABSTRACT

In *peste des petits ruminants* (PPR) affected goat / sheep flocks the most commonly occurring mixed viral infection is orf. Hence the present study was aimed to study the interaction of PPR virus (PPRV) and orf virus by co-infecting both the viruses *in vitro* using Vero cells. Both the PPRV field isolate and orf virus field isolate, adapted in Vero cells were utilized for co-infection. Equal quantum of both the viruses (100 $CCID_{50}$) was used for all the three methods of co-infection (Initial infection with PPRV virus followed by orf virus, Initial infection with orf virus followed by PPR Virus and Simultaneous infection with both the PPR virus and orf virus). Three types of control i.e. PPRV infected Vero cells, orf infected Vero cells and uninfected Vero cells were maintained. In all the three types of co-infection of PPRV and orf virus, the results revealed only the establishment of PPRV infection in Vero cells which was detected even at 5th passage whereas, the co-infected orf virus was not detected after second passage. Both the orf virus and PPRV were detected in all the five passages of their virus control Vero cells infected separately with respective viruses. The PPRV has dominated the co-infected orf virus and established the infection even at 5th passage in Vero cells.

Keywords: PPR, orf, Co-infection, *In vitro* studies

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INTRODUCTION

Peste des petits ruminants virus (PPRV) causes acute contagious disease in goats and sheep. PPRV is classified as a member of the genus *Morbilivirus* that comes under the family *Paramyxoviridae* of the order *Mononegavirales* (International Committee on Taxonomy of Viruses, 2017). PPR usually occurs year round, though an association with season has been observed (Abubakar *et al*., 2009). The clinical disease may be complicated by secondary infection with other pathogens such as those caused by *Pasteurella* spp., *Escherichia coli* and *Mycoplasma* spp. (Wohlsein and Saliki, 2006). Based on clinical signs, PPR may be confused with other diseases like capripox (CP), bluetongue (BT), contagious pustular dermatitis/ contagious ecthyma (orf), foot and mouth disease (FMD) and contagious caprine pleuropneumonia (CCPP). Dual infections can occur with other viruses such as pestivirus or GPV (OIE, 2012).

Concurrent infection of bluetongue virus (BTV) and PPRV in small ruminants was reported from Haryana State of India (Maan *et al*., 2017). Evidence of mixed infection of PPRV and BTV in a flock of goats was confirmed by detection of antigen, antibody and nucleic acid of both the viruses (Mondal *et al*., 2009). Presence of mixed infection of GPV with PPRV has also been reported (Malik *et al*., 2011). Mixed infection of PPR and orf on a goat farm was reported in Shahjahanpur,

***Corresponding author E-mail id: giridharanpalani@gmail.com**

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India. In that farm, morbidity due to PPR and orf was 63.2% (110 of 174) and 5.7% (10 of 174), respectively, while that due to mixed infection it was 11.5% (20 of 174). Overall, a mortality of 37.35% (65 of 174) was recorded, which was mainly attributed to PPRV infection and partly to the severity of the oral lesions exaggerated by ORFV infection. Approximately 20 goats had abortions (Saravanan *et al.*, 2007). Orf was observed in goats and sheep affected with PPR in the same flock and also in the same animal. Another important observation was the detection of orf in goats and sheep affected with PPR during recovery stage, usually second week after infection (Saravanan *et al.*, 2007).

Based upon the infection time, mixed infection is classified as co-infection when both the viruses infect simultaneously or, superinfection when one virus invades the host prior to the second virus. Viral interference is a phenomenon whereby one virus inhibits replication of other viruses. If both the viruses belong to the same family, the interference is referred to as homologous viral interference, whereas within the same species but different serotype, it is referred to heterotypic interference. Among viruses of different families, it is referred to as heterologous interference (Kumar *et al.*, 2016).

Deeba *et al.* (2016) has studied co-infection with Dengue and Chikungunya viruses. Co-infection of the two viruses was studied *in vitro* also using *Aedes albopictus* C6/36 cell line. In this study, the DENV-3 and Chikungunya virus (ECSA genotype) isolated from the infected mosquitoes were used to characterize their co-infection. The duplex-RT-PCR (DRT-PCR) technique was used to determine virus production.

Carillo-Tripp *et al.* (2016) have also studied *in vivo* and *in vitro* infection dynamics of honey bee viruses. The honey bee (*Apis mellifera*) is commonly infected by multiple viruses. They developed an experimental system for the study of such mixed viral infections in newly emerged honey bees and in the cell line AmE-711, derived from honey bee embryos. When inoculating a mixture of iflavirids [sacbrood bee virus (SBV), deformed wing virus (DWV)] and dicistrovirids [Israeli acute paralysis virus (IAPV), black queen cell virus (BQCV)] in both live bee and cell culture assays, IAPV replicated to higher levels than other viruses despite the fact that SBV was the major component of the inoculum mixture.

The vectors for transmission of dengue and chikungunya are *Aedes aegypti* and *Aedes albopictus* in mosquitoes. Since both dengue and chikungunya viruses are transmitted through a common vector, they often co-circulate in the mosquito and are transmitted to human beings as co-infections following the mosquito bite. It may not always be easy to differentiate the two infections clinically due to overlap of many symptoms. There is a rise in the number of these cases during and subsequent to the monsoon months. The climatic conditions during this period favour vector breeding places and thereby increasing the number of mosquitoes; resulting in a spurt of malaria, dengue and chikungunya cases. There has been a similar trend in the earlier studies reported from India. The prevalence of co-infection using serological methods has been reported in the earlier studies as 2.7%, 2.8% and 12.4%. The first study comparing the clinical, serological profile and molecular diagnosis of co-infected DENV and CHIKV patients with mono infection of either viruses from Western India was conducted by Londhey *et al.* (2016) and they reported 6.7% of co-infections.

Kumar *et al.* (2016) have stated that the occurrence of multiple virus infections is ubiquitous in natural populations, which may have significant epidemiological and biological effects. It is most commonly observed in immune-compromised individuals such as those infected with human immunodeficiency virus type 1 (HIV-1). Among the acute viruses, respiratory syncytial virus (RSV) and influenza virus infection in humans has been the most commonly reported mixed Infection (Kumar *et al.*, 2016). PPRV and orf virus, PPRV and BTV, PPRV and other respiratory viruses, PPRV and goat pox virus, PPRV and Border disease virus, PPRV and Rift Valley fever virus, are some of the mixed infections that have been observed in animals. The evidence of

multiple virus infection in most of these studies were determined by non-culture methods (serology/genome detection), however, isolation and purification of virus, particularly more than one virus, has not been well documented. Orf is the most common mixed viral infection that occurs in the goats / sheep flocks affected with PPR. Hence the present study was aimed to study the interaction of PPRV and orf virus by co-infecting both the viruses *in vitro*.

MATERIALS AND METHODS

Field investigation of the incidence of PPR in 176 flocks of goats and sheep from 2015 to 2017 showed positivity for 131 samples for PPRV. During the study of that period, 6 cases of bluetongue and 11 cases of orf were observed as inter-current infections with PPR. Inter-current infection with goat pox and sheep pox was not noticed. Attempts were not made to detect FMD during the study. However, few cases of inter-current infection with FMD in goats and sheep were observed.

The scabs were collected from goats and sheep for orf virus isolation and identification from eleven flocks of goats and sheep that occurred as inter-current infection during the incidence of PPR. The orf affected goats and sheep showed scabs on the lips, below muzzle and buccal commissures mostly from the animals recovering from PPR. Orf was detected by B2L gene analysis from 7 samples and virus from one sample was isolated.

Methods outlined in Carrillo–Tripp *et al.* (2016); Deeba *et al.* (2016) and Kumar *et al.* (2016) were followed with modification. Vero cells were selected for co-infection study of PPRV (PPRV/002/G/2015) and orf (Orf/G/002/ 2017) virus because both the viruses can be adapted and grown in this cell line. Field isolates of both the PPRV and orf viruses adapted in Vero cells were used for co-infection.

1. When Vero cells were 80% confluent, the supernatant media was discarded and in the first set of co-infection, 1ml of PPRV isolate (100 $CCID_{50}$) was added first and incubated for 30 min. The first supernatant was removed and then 1ml of Vero cell culture adapted orf virus (100 $CCID_{50}$) was added subsequently and incubated for 30 min. The supernatant was then removed. The Vero maintenance medium was added and the flasks were incubated at 37°C.
2. The same procedure was adopted for second set of co-infection in which orf virus was incubated for half an hour and removed, then PPRV virus was incubated subsequently for half an hour and removed.
3. In the third set, 0.5 ml (100 $CCID_{50}$) each of both PPRV virus and orf virus were added together and incubated at 37°C for 1 hour and then the supernatant was removed and maintenance medium was added.
4. Three types of controls i.e. PPRV infected Vero cells, orf infected Vero cells and uninfected Vero cells were maintained.

For PPRV and orf virus infected Vero cells controls, when Vero cells were 80% confluent, the supernatant media was discarded from designated cell culture flasks for separate viruses and 1ml (100 $CCID_{50}$) of respective virus isolate was added in respective flask and incubated at 37°C for 1 hour. The supernatant was then removed. The Vero maintenance medium was added and the flasks were incubated at 37°C. For uninfected Vero cells, maintenance medium was used as inoculum and incubated at 37°C for 1 hour. The Vero maintenance medium was added subsequently and the flasks were incubated at 37°C.

5. As the infection progressed, the medium became acidic and hence one or two drops of sterile 8.8% sodium bicarbonate solution was added to keep the medium with pH 6.5-7.0. The infected cells were observed daily for any change or cytopathic effect (CPE) for 5 days.
6. The infected monolayers in the 25 cm^2 flasks were frozen to -80°C and subsequently thawed at 37°c. Three cycles of freezing and thawing were carried out before inoculating the supernatant into the cells for next passage.
7. This inoculum was used for subsequent 4 passages.

Vero cell supernatants of all the five passages from the three types of co infections and Vero cell control infected only with PPR virus were subjected to RT PCR by using N gene primers for identification of PPRV. RNA was extracted using Trireagent (Sigma) and Qiagen 1 step RT-PCR kit was used to amplify 351 bp product of partial N gene using the forward primer NP3 (1232-1255) - 5′GTCTCGGAAAsTCGCCTCACAGACT3′ and the Reverse primer NP4 (1583-1560)-5′CCTCCTCCT GGTCCTCAAGAATCT3′ as per the method outlined by Couacy-Hymann *et al.* (2002) and OIE (2012). The PCR reaction was carried out with the following thermal cycling conditions:

Reverse transcription	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Hold
50°C	95°C	94°C	60°C	72°C	72°C	4°C
30 min	5 min.	30 sec.	30 sec.	1 min.	5 min.	Indefinite
1 cycle	1 cycle	40 cycles			1 cycle	

The supernatants as mentioned above for all the five passages of three types of co-infections and Vero cell controls infected only with orf virus alone were subjected to PCR by using B2L gene primers for identification of orf. DNA extraction from the supernatants were carried out using QIAamp DNA Mini Kit (51306) as per the protocol and final elution of DNA was done in 30 µl of elution buffer.

The extracted DNA was subjected to polymerase chain reaction (PCR) using Amplicon Red dye master mix in Eppendorf Vapoprotect PCR machine to amplify 1137 bp product of orf virus B2L gene with the primers OVB2LF1-5′ TCCCTGAAGCCCTATTATTTTTGTG3′ and OVB2LR1-5′ GCTTGCGGGGGTTCGGACCTTC3′ as per the method outlined by Hosamani *et al.* (2006). The PCR reaction was carried out with the following thermal cycling conditions:

Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Hold
94°C	94°C	52°C	72°C	72°C	4°C
3 min.	1 min.	1 min.	1 min.	10 min.	Indefinite
1 cycle	30 cycles	1 cycle			

RESULTS AND DISCUSSION

The RT-PCR results of PPRV N gene amplification from the supernatant of the co-infected Vero cell cultures with initial infection with PPR virus followed by orf virus at passage 1, 2, 3, 4 and 5 are presented in Table 1. The PCR results of orf virus B2L gene amplification from the same set of supernatants for the consecutive five passages are presented in Table 1.

In the similar way, results of RT-PCR of PPRV N gene amplification and PCR of orf virus B2L gene amplification from the supernatants of other four type of infection such as initial infection with orf virus followed by PPR Virus, simultaneous infection with PPR virus and orf virus, infection with PPR virus alone and infection with orf virus alone were also presented in Table 1 and shown in Fig. 1 and 2.

In the present study, equal quantum of both the viruses (100 $TCID_{50}$) was used for all the three methods of co-infection. In all the three types of co-infection of PPRV and orf virus the results revealed only the establishment of PPRV infection in Vero cells which was detected even at 5^{th} passage whereas the infection of Vero cells with orf virus was not detected from second passage onwards. But orf virus was detected in all the five passages of control vero cells infected with orf virus alone.

Deeba *et al.* (2016) has studied co-infection with Dengue and Chikungunya viruses in vitro also using *Aedes albopictus* C6/36 cell line. In their study, the DENV-3 and Chikungunya virus (ECSA genotype) isolated from the infected mosquitoes, when subjected to the duplex-RT-PCR (DRT-PCR) technique to determine their production, showed positive result for both the viruses at an equal multiplicity of infection (MOI) or a higher MOI for Chikungunya virus. But when a higher titer of DENV- 3 was used, the D-RT-PCR was positive only for DENV-3. Thus, the authors concluded that higher titer of Dengue-3 virus resulted in competitive suppression of the replication of Chikungunya virus. They also reported that replication of both the viruses depend on virus titer and not on serial infection.

Table 1: Genomic detection of PPRV and orf virus after co- infection in Vero cells

Vero cell infection	Passage 1		Passage 2		Passage 3		Passage 4		Passage 5	
	PPRV N gene RT-PCR	Orf B2L gene PCR	PPRV N gene RT-PCR	Orf B2L gene PCR	PPRV N gene RT-PCR	Orf B2L gene PCR	PPRV N gene RT-PCR	Orf B2L gene PCR	PPRV N gene RT-PCR	Orf B2L gene PCR
Initial infection with PPRV virus followed by Orf virus	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Initial infection with Orf virus followed by PPR Virus	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Simultaneous infection with PPR virus and Orf virus	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Infection with PPR virus alone	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Infection with Orf virus alone	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve

+ve: Positive; -ve: Negative

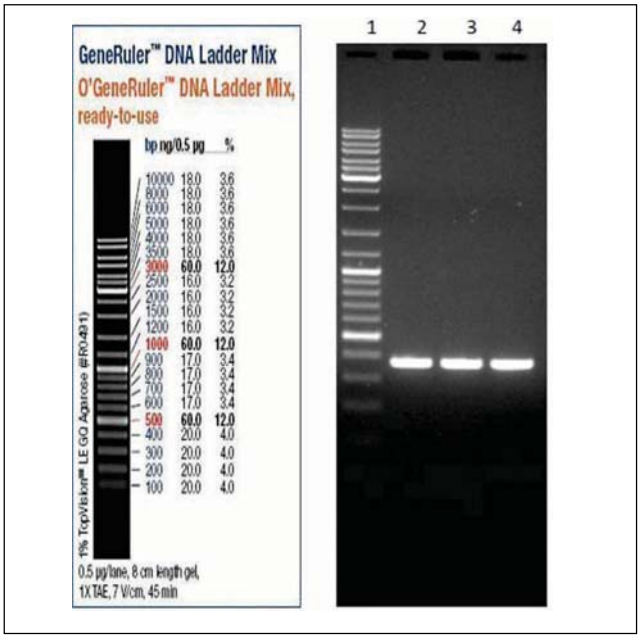

Fig. 1: RT-PCR amplification of N gene of PPRV
Lane 1 : 10 kb Marker (Thermo Scientific) while Lane 2, 3, 4 are 351 bp N gene specific amplicon of PPRV passage 1 for all three type co-infections

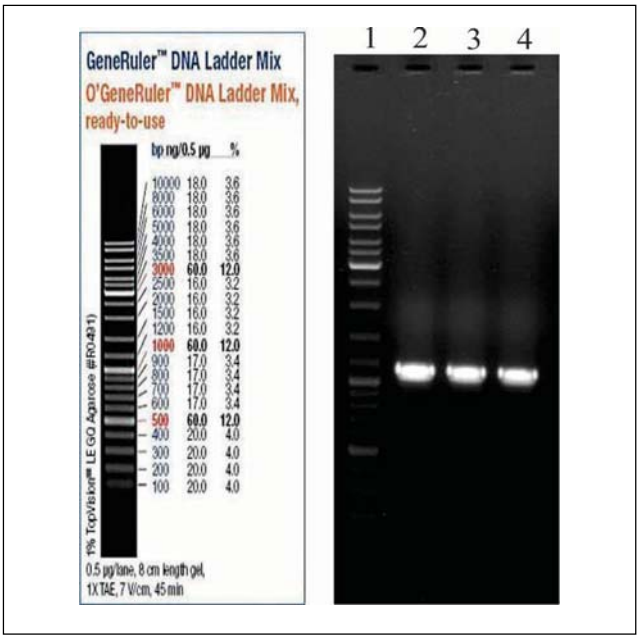

Fig. 2: PCR amplification of B2L gene of orf virus
Lane 1 : 10 kb Marker (Thermo Scientific) Lane 2 : 1137bp of B2L gene specific amplicon of orf passage 1 for all three type co-infections

Carillo-Tripp *et al.* (2016) have also studied *in vivo* and *in vitro* infection dynamics of honey bee viruses. The honey bee (*Apis mellifera*) is commonly infected by multiple viruses. The workers developed an experimental system for the study of such mixed viral infections in newly emerged honey bees and in the cell line AmE-711, derived from honey bee embryos by inoculating a mixture of iflavirids and dicistroviridae. Israeli acute paralysis virus (IAPV) replicated to higher levels than other viruses despite the fact that sacbrood bee virus (SBV) was the major component of the inoculum mixture. When a different virus mix composed

mainly of the dicistroviridae Kashmir bee virus (KBV) was tested in cell culture, the outcome was a rapid increase in KBV but not IAPV. They also found that when deformed wing virus (DWV) covertly infects the AmE-711 cell line the virus does not prevent IAPV and KBV from accumulating to high levels and causing cytopathic effects. These results indicate that different mechanisms of virus-host interaction affect virus dynamics, including complex virus-virus interactions, superinfections, specific virus saturation limits in cells and virus specialization for different cell types.

Kumar *et al*. (2016) investigated PPRV and foot-and-mouth disease virus (FMDV) mixed infection in goats. Rather than in a single cell type, cytopathic effect (CPE) of the virus was observed in cocultured Vero/BHK-21 cells at 6th blind passage (BP). Mixed infection was not found to induce any significant antigenic and genetic diversity in both PPRV and FMDV. Further, it was demonstrated that the viral interference between PPRV and FMDV resulted in reduced FMDV replication in BHK-21 cells suggesting that the PPRV RNA induced interference was specifically directed against FMDV. On long-term coinfection of some acute pathogenic viruses in Vero cells, in most cases, one of the co infecting viruses was excluded at passage level 5 suggesting that the long-term coinfection may modify viral persistence. The coinfection may result in either coexistence (viral accommodation) of both the viruses or elimination (virus exclusion) of one and survival of the other (persistence)

In the current study of co-infection of PPRV and orf virus in vitro, the PPRV has dominated and established the infection in Vero cells even at 5th passage. Establishment of infection by PPRV could be due to its ssRNA nature. Hence, more detailed studies are warranted by studying the cytokines using real time PCR and confocal microscopy to further elucidate intricacies of co-infection involving PPRV and orf virus in *vitro* and *in vivo*.

CONCLUSION

The study was aimed to assess the interaction of PPR virus (PPRV) and orf virus in vitro by co-infecting the viruses in Vero cells. Field isolates of both the viruses, adapted in Vero cells, were used for co-infection. After serial passages of co-infection of these viruses in Vero cells, the PPRV was found to dominate over the orf virus in infecting and getting established in the cells, which was revealed by its detection even at 5th passage, whereas, the orf virus could not be detected after second passage. Both the orf virus and PPRV were detected in all the five passages of their virus control Vero cells infected separately with respective viruses. It is speculated that establishment of infection by PPRV could be due to its ssRNA nature. Further studies are required to elucidate their interaction after co-infection in vivo in laboratory animals/ natural host in establishing infection in various organs/tissues, their pathogenesis and immunology.

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REFERENCES

Abubakar, M., Jamal, S.M., Arshed, M.J., Hussain, M., Ali, Q. (2009). *Peste des petits ruminants* virus infection; its association with species, seasonal variations and geography. *Trop. Anim. Health Prod.*, 41: 1197–1202.

Carrillo–Tripp, J., Dolezal, A.G., Goblirsh, M.J., Miller, W.A., Toth, A.L., Bonning, B.C. (2016). *In vivo* and *in vitro* infection dynamics of honey bee viruses. *Scientific Reports*, doi*:10.1038./srep22265*: 1-12.

Couacy-Hymann, E., Roger, F., Hurard, C., Guillou, J.P., Libeau, G., Diallo, A. (2002). Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. *J. Virol Methods.*, 100: 17–25.

Deeba, F., Afreen, N., Islam, A., Naqvi, I.H., Broor, S., Ahmed, A., Parveen, S. (2016). Co-infection with Dengue and Chikungunya Viruses. *INTECH open Science/open Minds*, 6: 105-119, http://dx.doi.org/10.5772/64308.

Hosamani M., Bhanuprakash, V., Scagliarini, A., Singh, R.K. (2006). Comparative sequence analysis of major envelope protein gene (B2L) of Indian orf viruses isolated from sheep and goats. *Vet. Microbiol.*, 116: 317-24. 10.1016/ j.vetmic.2006.04.028.

International Committee on Taxonomy of Viruses (ICTV). (2017). Virus taxanomy: classification and the nomenclature for

viruses. *Virus Taxonomy: 2017 Release EC*49, Singapore, July 2017.

Kumar, N., Barua, S., Riyesh, T., Chaubey, K.K., Rawat, K.D., Khandelwal, N., Sharma, N., Sharma, S., Chandel, S.S., Sharma, S., Singh, M.K., Sharma, D.K., Singh, S.V., Tripathi, B.N. (2016). Complexities in isolation and purification of multiple viruses from mixed viral infections: viral interference, persistence and exclusion. *PLoS ONE*, 11: 1-24, e0156110.doi:10.1371/journal.pone. 0156110.

Londhey, V., Agrawal, S., Vaidya, N., Kini, S., Shastri, J.S., Sunil. S. (2016). Dengue and Chikungunya Virus Co-infections: The Inside Story. *J. Assoc. Physicians India*, 64: 36-40.

Maan, S., Kumar, A., Gupta, A.K., Dalal, A., Chaudhary, D., Gupta, T.K., Bansal, N., Kumar, V., Batra, K., Sindhu, N., Kumar, A., Mahajan, N.K., Maan, N.S., Mertens, P.P.C. (2017). Concurrent infection of bluetongue and *peste des petits ruminants* virus in small ruminants in Haryana State of India. *Transbound. Emerg. Dis.*, pp 1–5. doi: 10.1111/tbed.12610.

Malik, Y.S., Singh, D., Chandrashekar, K.M., Shukla, S., Sharma, K., Vaid, N., Chakravarti, S. (2011). Occurrence of dual infection of peste *des petits ruminants* and goat pox in indigenous goats of central India. *Transbound. Emerg. Dis*., 58: 268-272.

Mondal, B., Sen, A., Chand, K., Biswas, S.K., De, A., Rajak, K.K., Chakravarti, S. (2009). Evidence of mixed infection of *peste des petits* ruminants virus and bluetongue virus in a flock of goats as confirmed by detection of antigen, antibody and nucleic acid of both the viruses. *Trop. Anim. Health Prod.*, 41: 1661-7.

OIE, World Organisation for Animal Health. (2012). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th Edition, Vol 1 and 2 (Version adopted in May 2013). Chapter on *peste des petits ruminants.*

Saravanan P, Balamurugan, V., Sen, A., Sarkar, J., Sahay, B., Rajak, K.K., Hosamani, V., Yadav, M.P., Singh, R.K. (2007). Mixed infection of *peste des petits ruminants* and Orf on a goat farm in Shahjahanpur. *Indian Vet. Res.*, 160: 410–412.

Wohlsein, P., Saliki, J. (2006). Rinderpest and *peste des petits ruminants* virus: Plague of Large and Small Ruminants; Biology of Animal Infections. *Academic Press: London, UK*, pp 69–85.

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