

# Study of Peste Des Petits Ruminants (PPR) in Some Border Areas of Iran by Nested- PCR

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## Abstract

In order to study on Peste Des Petits Ruminants (PPR) in some border areas of Iran, totally 70 blood samples were taken via jugular vein from Kamyaran, Piranshahr, Lahijan, Ghorveh and Bilesavar district, in a tube with EDTA. The sampling was in the flocks located in Iran borders. The samples were sent near the ice container to laboratory. The blood samples included 28 goat and 42 sheep which totally consisted of 45 and 25 female and male respectively. Samples collected from Ghorveh, Lahijan, Billesavar, Kamyaran and Piranshahr were 13, 10, 18, 13, and 16 respectively. In the above mentioned areas, non-specific symptoms of PPR such as high mortality rate, diarrhea that resistant to the treatment, abortion and observation of ulcer in the penis of the male animals were observed. Sampling was not only from suspected animals but also was done with animals with no specific clinical signs (apparently normal sheep and goats). Blood samples centrifuged at 4° C in 3000 rpm for 10 minutes and serum was separated quietly. Buffy coat was used for extracting PPRV (RNA). RT-PCR was performed by using 448bp that is specific for F gene. In the second step, all samples were tested with Nested-PCR. Live attenuated vaccine was used as a positive control in PCR. Statistical analysis of data was done by SPSS and analysis of variance. Out of 70 samples, N=10 (15%) were positive in the PCR tests. The infection in sheep and goat was N=4 (%10) and N=6 (%22) respectively. No significant differences ( $P>0/05$ ), were observed between the gender and infection. In the age group of less than 1, 1-2, 2-3 and over 3 years-old, infection rate was %14, 5 %34, 2 %12 and %0, respectively presenting no significant difference ( $P>0/05$ ). The infection rate in Ghorveh, Lahijan, Billesavar, Kamyaran and piranshahr, was %30, %0, %30, %20 and %20, respectively. The analysis showed that there were no significant differences between the flocks in the locations ( $P>0/05$ ). Other studies are suggested to be done in different borders of Iran to distinguish infection of PPR.

**Key words:** PPR, Sheep, Goat, Iran

## Introduction

Peste des petits ruminants (PPR) is a highly contagious viral disease of sheep and goat which was first reported in West Africa in early 1940s (Gargadennec and Lalanne, 1942). The PPR belongs to genus Morbillivirus in the family of

Paramyxoviridae (Gibbs and Taylor, 1979). Morbilli viruses are antigenically related to viruses such as: Measles, Rinderpest and Canine Distemper virus (Barrett *et al.* 1996, Bazarghani *et al.* 2006). This disease occurs in two forms of acute and sub-acute (Anderson and Mckay, 1994) in domestic and wild ruminants and includes these clinical signs:

high fever, ocular-nasal discharge, pneumonia, necrosis stomatitis, enteritis and diarrhea (Gibbs *et al.* 1979). This disease is one of the major reports of World Organization for Animal Health (OIE) (Lefevre and Diallo 1990). In areas where it happens in epizootic form, it makes wretched lesions, so that morbidity rate rises up to 90% and mortality rate increases up to 80% (Lefevre & Diallo 1990). Goat is more sensitive than sheep (Lefevre & Diallo 1990). It does not have any cure but prevention and control is effective. It has been a while that PPR like other countries of middle East conquered the borders of Iran. Due to this, highly important disease in animal health and economy, reviewing its various aspects is essential. In addition, Iran is a neighboring country to Iraq, Turkey, Afghanistan and Pakistan which are major centers of animal and human diseases (Toplu, 2004). PPR is confirmed with virus isolation (Bazarghani *et al.* 2006). Due to importance of differentiating PPR with other diseases which have the same clinical signs such as Rinderpest, laboratory test should be conducted. These tests can detect virus's antibody or antigen in the sample. In this study, Nested-PCR was used to detect the presence of PPR virus in border areas and all of the tests were conducted in Virology lab of Faculty of Veterinary Medicine of University of Tehran. This disease is highly contagious when it first happens in native population. Morbidity rate reaches 90% and mortality rate reaches 50% to 80% (Abubakr.M *et al.* 2008). In areas where PPR is enzootic, its prevalence is low and usually in these areas, animals become infected every 2 to 3 years. It occurs mostly in infants and not much in elders. Between various techniques of detecting PPRV, PCR with using primers of gene F is the most

acceptable technique for diagnosis and epidemiological studies (Brindha *et al.* 2001). RT-PCR with general primer of phosphoprotein (P) and some special primers of gene F was introduced by Barrett *et al.* 1993 and Couacy *et al.* 2002 to diagnose and differentiate PPR from RP.

### Materials and Methods

Seventy sheep and goats (48 sheep and 22 goats) were selected from border areas of Iran regardless of their breed, age and sex. Five ml of blood obtained from each animal using Venujects (Mediplus-K2EDTA-4ml China) was sent to laboratory near ice. The reason for selecting these border areas was due to the presence of signs like extreme mortality rate, resistance to cure, abortion and lesions in penile mucosa; however, all of the samples were taken from visually healthy animals. In the laboratory, samples were centrifuged in 4°C with 3000 r/min for 10 minutes; then, serum was isolated and stored in -20°C during the test time. After centrifuging, the middle part of samples which contained Buffy coat was used to extract PPR virus's RNA.

#### Areas which were studied:

- 1) Gorveh, is in Kordestan province between Sanandaj and Hamedan. (Latitude 35 and Longitude 47).
- 2) Rood Boneh, part of Lahijan city from Gilan Province (Latitude 37 and Longitude 50).
- 3) Piranshahr, in southern part of west Azerbaijan province which has borders with Iraq (Latitude 36 and Longitude 45).

- 4) Border town of Kamyaran in Kordestan province (Latitude 34 and Longitude 35).
- 5) Bilesavar town in Ardabil province which has borders with Azerbaijan from west and North-West parts (Latitude 39 and Longitude 48).

#### **RNA extraction**

The RNA was extracted from blood Buffy coats using the viral gene-spin kit (Intron Biotechnology, South Korea) according to the manufacturer's instructions. Briefly, 300  $\mu$ L of blood buffy coats and 500  $\mu$ L lysis buffer were mixed by vortex. After adding proteinase-k, samples were incubated at 55 °C for 10 min and centrifuged for 1 min at 13,000 rpm. A volume of 700  $\mu$ L binding buffer was added and shaken gently; then, 500  $\mu$ L washing buffer-A was added to suspension and centrifuged for 1 min at 13,000 rpm. This step was repeated by washing buffer-B. Finally, 30  $\mu$ L elution buffer was added and after centrifuging, extracted nucleic acid was collected.

#### **Reverse transcription**

Reverse transcription was performed on ribonucleic acids (RNA) extracted from blood buffy coats by 2-steps RT-PCR kit (Vivantis, Malaysia). A volume of 8  $\mu$ L RNA, 1  $\mu$ L random hexamer primer (50 ng concentrations) and 1  $\mu$ L dNTP mix (10 mM) were mixed and incubated in 65 °C for 5 min and were placed on ice. Then, 0.5  $\mu$ L M-MuLV reverse transcriptase enzyme (100 unit) and 2.5  $\mu$ L of 10X Buffer M-MuLV and 7.5  $\mu$ L nuclease-free water were added and placed in 42 °C for 60 min and 85 °C for 10 min.

#### **RT-PCR and Nested RT-PCR**

Reverse transcriptase polymerase chain reaction (RT-PCR) and Nested RT-PCR were performed by the method suggested by Forsyth and Barrett, (1995). Primer sequences were targeted for F gene and sequences were: PPRV F1 5'-ATCACAGTGTAAAGCCTGTAGAGG-3' at position 777-801 and PPRV F2 5'-GAGACTGAGTTTGTGACCTACAAGC-3' at position 1124-1148 and primers for second step of Nested PCR were: PPRV F1A 5'-ATGCTCTGTCAGTGATAACC-3' at position 802-821 and PPRV F2A 5'-TTATGGACAGAAGGGACAAG-3' at position 1092-1110 which amplify specific 371 and 308 bp PCR products, respectively. Briefly, 2.5  $\mu$ L of 10X PCR buffer, 0.75  $\mu$ L MgCl<sub>2</sub> (50 mM), 0.25  $\mu$ L *Taq* DNA polymerase, 1  $\mu$ L dNTP mix (10 mM) and 1  $\mu$ L from each primers (10 mM) were added and then total volume reached to 22  $\mu$ L with distilled water. Finally, 3  $\mu$ L cDNA or PCR product (for Nested RT-PCR) was added to it and was placed in the thermocycler. For RT-PCR, after an initial denaturation period of 5min at 95 °C, reactions were subjected to 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C and final extension of 7 min at 72 °C. Then, RT-PCR products were subjected for Nested RT-PCR with condition including an initial denaturation period of 5min at 95 °C, then 30 cycles of 1 min at 94 °C, 1 min at 65 °C and 1min at 72 °C and final extension of 10 min at 72 °C. Each 10  $\mu$ L of reaction products with 2  $\mu$ L of loading buffer were electrophoresed through 1.5% agarose gel and were stained with ethidium bromide. The appropriate molecular weight markers (100-bp DNA ladder; Sinaclon, Karaj, Iran) were

used. The positive control included the extracted nucleic acid of the commercial strains of the vaccine (Nigeria 75.1, Spain) and the negative control consisted of all the RT-PCR/PCR reagents except for the nucleic acid; these were included in each reaction (Forsyth and Barrett, 1995). Evaluation of polymerase chain reaction for the detection and characterization of rinderpest and peste des petits ruminants viruses for epidemiological studies was done. The statistical analysis of data was performed with SPSS V.17. Ratio comparison and Chi-square

test were used to find the significant relation between data.

### Results

In this study, seventy blood samples of sheep and goat from various areas of Iran were collected. From all of these samples, 34 were from Bilesavar and Piranshahr which were (taken from suspicious animals) and 36 were from Gorveh, Lahijan and kamyaran (taken from visually healthy animals). From 34 suspicious samples, 5 were positive and from 36 visually healthy samples 5 were positive (Table 1-3).

Table 1: Total number of samples based on sex and area of study

Breed & Sex		Lahijan	Gorveh	Bilesouvar	Kamyaran	Piranshahr	Total	Percent
Sheep	Female	4	5	10	0	10	29	41.42
	Male	3	5	2	0	3	13	18.57
Goat	Female	2	1	4	6	3	16	22.85
	Male	1	2	2	7	0	12	17.14
Total		10	13	18	13	16	70	100

Table 2: Results of disease study in goat based on age

Results		Under 1 year old	1 to 2 years old	2 to 3 years old	upper than 3 years old	Total	Percent
Negative	Female	5	3	2	3	13	46.42
	Male	2	1	1	5	9	32.14
Positive	Female	2	1	0	0	3	10.71
	Male	0	2	1	0	3	10.71
Total		9	7	4	8	28	100

Table 3: Results of disease study in sheep based on age

Results		Under 1 year old	1 to 2 years old	2 to 3 years old	upper than 3 years old	Total	Percent
Negative	Female	8	3	8	7	26	61
	Male	4	4	4	1	12	28.57
Positive	Female	1	2	0	0	3	7.14
	Male	0	0	1	0	1	2.38
Total		13	8	13	8	42	100

Among 45 samples from female animals, 6 were positive (13.33%) and from 25 samples from male animals, 4 were positive (16%); so, no significant relation was found between sex and infection. The infection rate based on animal age is illustrated in figures 3-3 and 3-4. To check the relation between age and infection rate, samples were divided into 4 groups: Under one-year-old, one to two years old, and two to three years old and upper than three. The rates of infection in these groups were respectively: %14, %34, %12, %0. But in Chi-Square test, no significant relation was observed between age and infection ( $P>0.05$ ). In figure 3-5 the total number of positive cases is

demonstrated. No significant relation was observed concerning the area of the study either. Most positive cases happened in Gorveh and Bilesouvar (3 positive samples= 30%) and the least positive cases were from Lahijan (0%). There was no significant relation between the study areas and the positive results ( $P>0.05$ ). In figure 1-5, abundance and percentage of positive cases are illustrated respectively based on the study areas, which are generally shown in table 1-3.

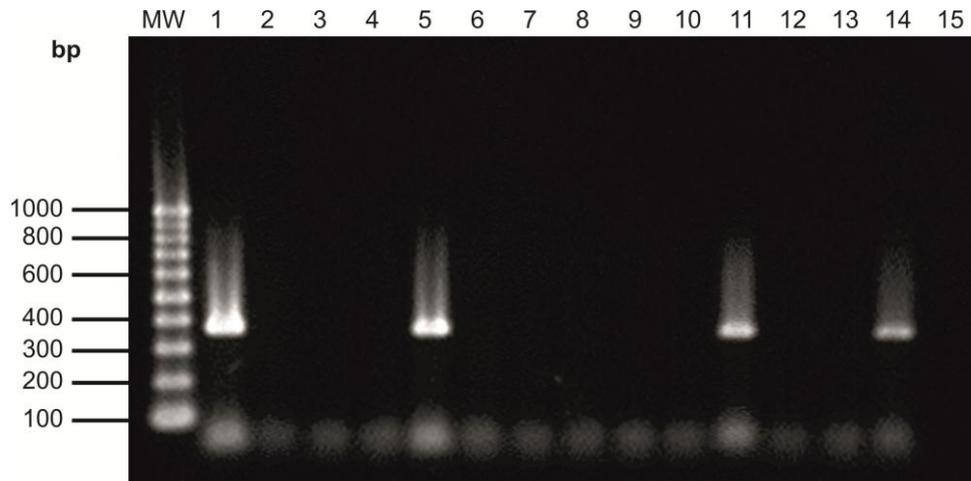


Figure 1: Results of multiplication of Gene F of PPR virus from sheep and goat blood samples Gorveh. MW is 100 pb., 1 positive control, 5, 11 and 14 positive samples, well 15 is negative control and well numbers 2, 3, 4, 6, 7, 8, 9, 10, 12, 13, are negatives.

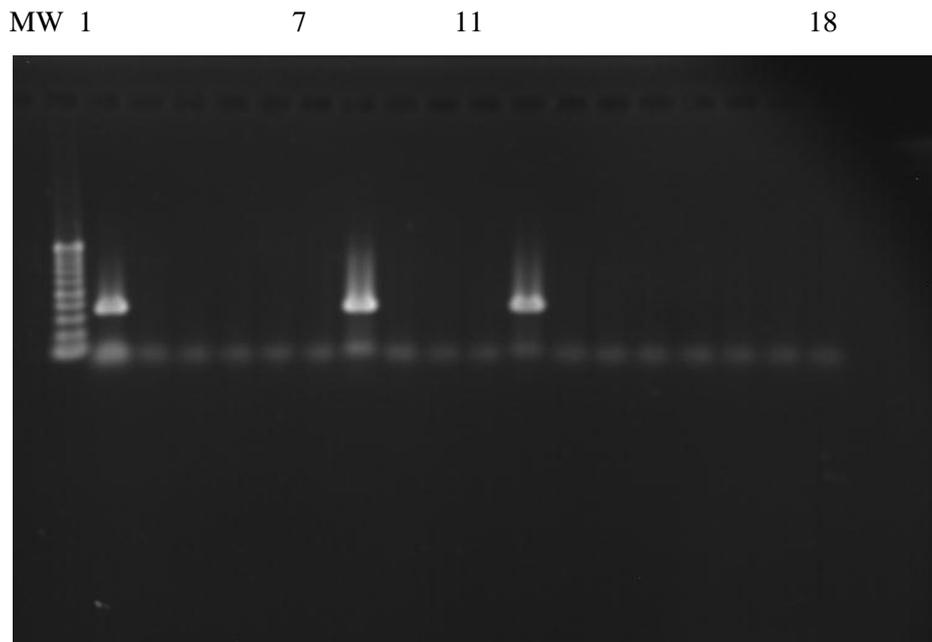


Figure 2: Results of multiplication of Gene F of PPR virus from sheep and goat blood samples of Piranshahr. MW is 100 bp., well number 1 is positive control., 7 and 11 and are positive samples, 18 well is negative and other numbers are negatives.

MW 1 7 11 14

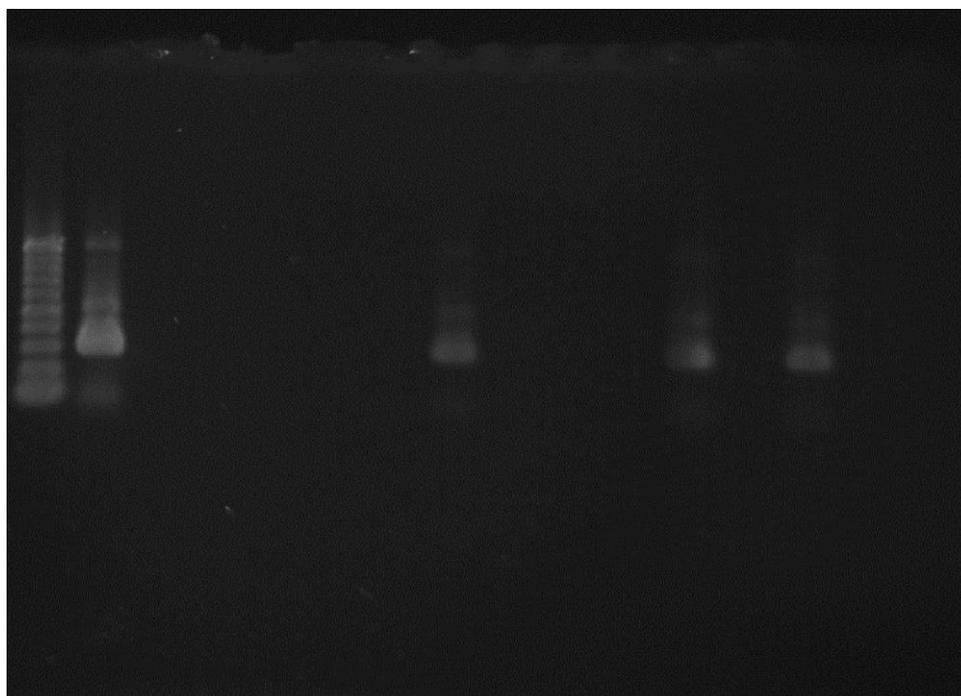


Figure 3: Results of multiplication of Gene F of PPR virus from sheep and goat blood samples of Bilesavar. MW is 100 bp., well number 1 is positive control. 7, 11 and 14 are positive samples. Other wells are negatives.

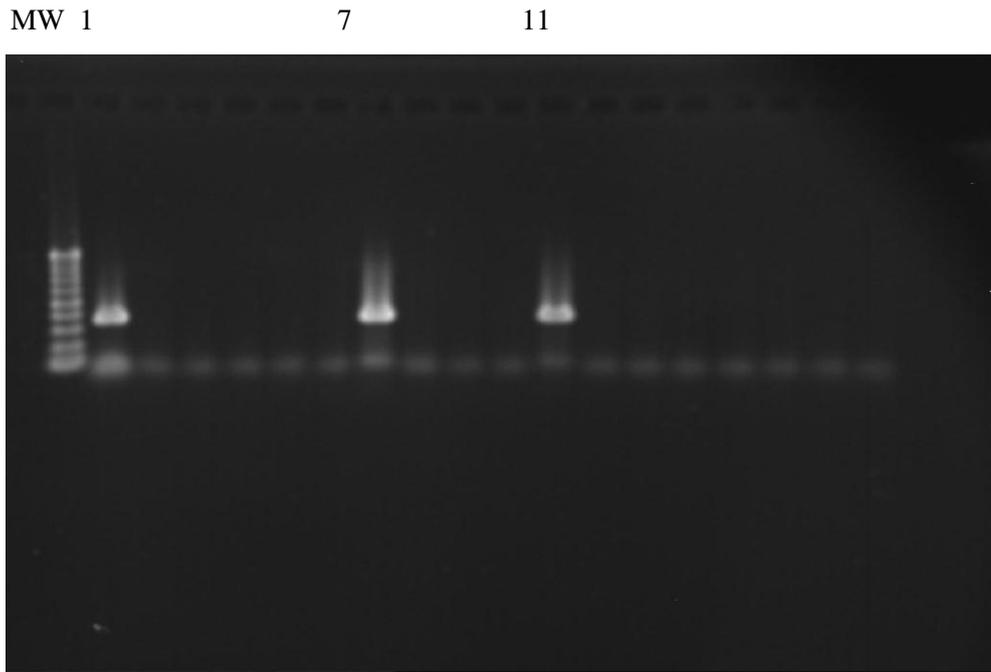


Figure 4: Results of multiplication of Gene F of PPR virus from sheep and goat blood samples of Kamiaran and Piranshar. MW is 100 bp., well number 1 is positive control. Well numbers 7 and 11 are positive. Others are negative for PPR.

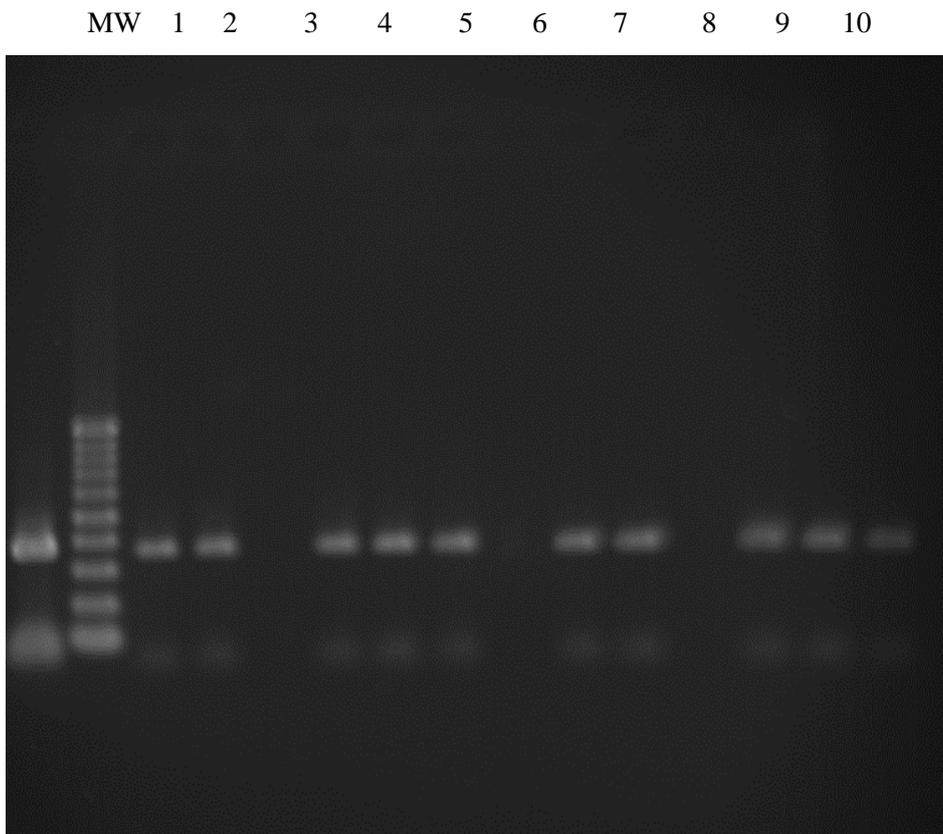


Figure 5: RT-PCR results in samples on Agarose jell. MW is 100pb. Well numbers 1 and 2 are positive from Kamiaran, 3, 4 and 5 positive samples from Gorveh, 6 and 7 positive samples from Piranshahr. Well numbers 8, 9 and 10 positive samples from Bilesavar.

## Discussion

Among laboratory diagnostic methods, Molecular techniques have the highest sensitivity and specialty in detection of morbillivirus like PPRV. In this study, Nested-PCR was used as a diagnostic tool to detect PPR. Blood samples from suspicious and visually healthy animals were first analyzed with RT-PCR. They were then tested with Nested PCR. The reason was to ensure the Nested-PCR results and to increase sensitivity of virus detection. Generally, Nested PCR is one of the simplest, fastest and in the meantime one of the most sensitive tests to confirm proliferated segments in PCR reaction. In the preset study, out of 42 sheep which were evaluated, 4 were positive (10%) and out of 28 goats, 6 were positive (22%). Out of 34 samples which were suspicious, 5 were positive and out of 36 samples which were visually healthy, 5 were positive. In the study of Mahajan *et al.* (2012), in India, after evaluating 432 serum samples with competitive ELISA, serum prevalence was 29.16% in sheep and was 28.70% in goat. In another study, by Munir *et al.* (2009) with ELISA, infection was 38.8% in sheep and was 25.6% in goat. Higher serum prevalence in sheep could possibly be because of its severe infection in sheep (Radostits, 2007). So, as a result of increasing serum prevalence, more infected sheep survive (Bhaskar *et al.* 2009). Shankar *et al.*'s (1998) study with ELISA indicates that being infected with this disease, mortality rate in sheep is less than goats and therefore, the sheep survive. Sharma *et al.* (2007) point out in their study that there is higher mortality rate in goats than sheep which have PPR. In the study conducted by Ahmad-Almajali *et al.* (2005) in

Jordan, out of 929 sheep blood sample and out of 400 goat blood sample, using competitive ELISA, 29% and 49%, respectively, were positive. In the aforementioned article, blood samples were taken from 122 different flocks in northern Jordan. Keeping sheep and goat together is a risky factor in which sheep gets the PPR infection but goat does not. PPR is endemic in Pakistan and has high morbidity and mortality rate. In Haider Ali-Khan's study, 933 serum samples in Panjab province were obtained from animals which had diarrhea and severe respiratory disease. They were tested with competitive ELISA to see if they had anti-PPR virus antigen. 51.34% of samples were positive ( $P < 0.432$ ) in small ruminants. The number of infected sheep was higher than goat (in sheep 56.8% and in goat 48.24%). In animals with more than 2 years, old prevalence of disease was higher than other age groups. Females were more infected than males. The reason for these findings may be due to different geographical areas, topography and social and financial status of farmers (Ali Khan *et al.* 2008).

However, in the current study, out of 45 female samples, 6 were positive (13.33%) and out of 25 samples from male animals, 4 were positive (16%). In Rahman *et al.*'s (2004) study, males were infected more than females which confirms the present study. Prevalence of PPR in Female goats is higher than males but the difference is little. But based on the study of Khan *et al.* (2008), it is significant. To study the correlation between age and infection rate, samples were divided 4 groups: Under one-year-old, one to two years old, two to three years old and upper than three. The most positive cases were in one-to-two year old group (33%) and the least

positive cases were in upper-than-three group (0%). In the study of Mahajan *et al.* (2012) samples were divided into three groups: 4 to 8 months, 8 to 12 months and upper than 12 months in which the highest serum prevalence in sheep was from "upper than 12 months" group (39.58%) and the least was from "4 to 8 months" (20.83%). Ozkol *et al.* (2002) indicated that animals smaller than six months old and more than one-year old are more likely to be facing disease. Taylor *et al.* (1979) mentioned that infection ages to this virus is 4 to 24 months and most of infection and mortality happens at this period. This finding was approved by others (Abu Elzein *et al.* 1990). Abubakar *et al.*, (2009) mentioned that with aging, prevalence of disease increases. In Ahmad *et al's* report (2005), it was mentioned that mortality rate in infants (one to three months old) compared to lambs (four to twelve months old) is higher and it is the same when lambs are compared to adults. No significant relation found when analyzing the data based on the study areas. Most positive cases were in Gorveh and Bilesouvar (3 positive samples=30%) and the least positive cases were in Lahijan (0%).

The experimental study conducted by Hymann *et al.* (2009) on goat reveals that with Immunocapture ELISA and RT-PCR, we can diagnose the illness before clinical signs arrive. He advises developed countries to use Immunocapture ELISA for early diagnosis of PPR. This disease was first reported in Ilam province which has borders with Iraq. In the same year, eight other provinces reported the illness got by 39 flocks (1995). From 1997 to 2004, the illness spread widely in Iran. During these years, there were different levels of morbidity and mortality. In the aforementioned period of time 1443 flocks were examined in which

Qom with 283 flocks and Semnan with 3 flocks had the most and the least number of infected flocks, respectively. Sheep and goats of Iran in that period of time were being vaccinated by cell cultured PPR vaccines and from 2005 on were being vaccinated with African PPR. From the third week of March to the third week of September 2005, 93 flocks which were infected by PPR in 16 provinces of Iran were determined; East Azerbaijan province had 19 infected flocks and Tehran and Chaharmahal Bakhtiari and Qazvin had one infected flock (Bazarghani *et al.* 2006). Despite vaccination and Controlling efforts, the disease has spread in Iran which may be due to the following reasons:

- 1) Higher sensitivity of Iranian goats and sheep
- 2) Absence of effective and good quarantine
- 3) Traditional behaviors of animal owners
- 4) Absence of vaccination

Because this illness reduces meat and milk and wool production, it needs a lot of attention (Bazarghani *et al.* 2006)

### **Suggestions**

In domestic areas, PPR is a major threat for keeping small ruminants. So it has major impact on farmers' financial status. As a consequence, controlling PPR is a major step in fighting against poverty. Considering that the virus is present at border areas of Iran, it is suggested that more complete studies be done to investigate the shape of illness in those areas. PPRV can infect other species

such as Cows and Camels; so, enough attention should be given to illness in these species especially in situations where the vaccination has been stopped. Additionally, it is suggested that, to control the illness, we should have increased vaccination efforts, controlled animal transportation and disinfection of disease centers.

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