The Journal of Animal & Plant Sciences, 27(1): 2017, Page: 91-97
ISSN: 1018-7081

CLINICAL, PATHOLOGICAL AND MOLECULAR DIAGNOSIS OF SHEEPPOX VIRUS IN SAUDI ARABIA

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ABSTRACT

The present study was aimed to asses clinical, histopathology, PCR and phylogenetic analysis of sheep pox virus (SPPV) in indigenous sheep. A sheep farm at Al Hassa District was struck by the sheep pox virus. The clinical signs were a sudden onset of fever, which peaks at 40–42°C, with discharges from the nose and eyes, and excessive salivation. The animal loses its appetite and is reluctant to move. Mortality was mostly seen in lambs. At necropsy, the skin lesions were erythematous macules and papules, measured 2 to 4 mm in diameter which may become larger and nodular. These lesions were frequently seen in face, back and tail. Multiple firm white nodules disseminated throughout the lobes of the lungs, particularly in lambs. Specific histopathologic features like epidermal hyperplasia with ballooning degeneration and the presence of intracytoplasmic eosinophilic inclusion bodies associated with hyperplasia of pneumocyte type II were seen in the affected animals. The collected samples were screened for the presence of SPPV DNAs using KS-1.5/KS-1.6 and InS-1.1/Ins-1.1/based multiplex PCR. The p32 gene of selected two positive samples was sequenced and aligned with different SPPV, GTPV and LSDV available in GenBank. The phylogenetic analysis revealed that sheep pox virus strain; SPPV/Al-Hassa/2014/Saudi Arabia (accession number, KP342531) clustered on SPPV clad with SPPVs from India and China.

Keywords: Sheep pox, Histopathology,PCR, Saudi Arabia.

INTRODUCTION

Sheeppox virus (SPPV) is a member of Capripox viruses (CaPVs) which belongs to the Poxviridae family (Fauquet et al., 2005). Various strains of the virus cause disease only in sheep (SPPV), others only in goats (GTPV), and some in both sheep and goats (Bhanuprakash *et al.,* 2010). Sheeppox virus (SPPV) has been recorded in Africa, the Middle East and Asia (Bhanuprakash *et al.,* 2006; Babuik *et al.,* 2008). In Saudi Arabia, Sheep pox is a serious problem and has been reported from different regions of the country (Abu-Elzein *et al.,* 2003; Abdulkarem *et al.,* 2014). Generally, sheep pox isa disease of considerable economic importance for indigenous sheep farming. Heavy economic losses in sheep pox outbreaks are due to mortality, abortions and loss of market value of the affected animals (Senthilkumar and Thirunavukkarasu, 2010; Rinku *et al.,* 2013). A risk factors of the disease, including age, sex, breed and physiological status of animal (Yashpal *et al.,* 1997; Senthilkumar *et al.,* 2006; Selvaraju, 2014). All age groups can be affected, however, mortality may be up to 50% in a fully susceptible flock and as high as 100% in young animals (Bhanuprakash *et al.,* 2005). Disease occurrence is also affected by ecosystem, physiography, soil types, rainfall and relative humidity and temperature (Bhanuprakash *et al.,* 2006). Sheeppox is characterized by fever, ocular and nasal discharges, papular dermatitis, and nodular lesions in a variety of organs, including the lungs, trachea, and the abomasums **(**Diallo and Viljoen, 2007). Histopathology was manifested by hyperkeratosis and dermatitis in skin with cytoplasmic inclusion bodies and the lung lesions are characterized by proliferation of type II pneumocyte with focal areas of neutrophils and lymphocytes (Gulbahar *et al.,* 2006; Beytut, 2010). Diagnosis of sheep pox is usually based on highly characteristic clinical signs (Ozmen et al., 2009), virus isolation, virus neutralization test, ELISA test (Tian *et al.,* 2010) and PCR assays (Balinsky *et al.,* 2008; Manjunatha *et al.,* 2015). However, conventional serological assays could not distinguish SPPV, GTPV and LSDV due to the close antigenic and virulence relationship (Balinsky *et al.,* 2008). Characterization of these viruses needs molecular techniques targeting CaPVs specific genes like P32, GPCR and RPO30 genes (Zhou *et al.,* 2012; Yan *et al.,* 2012).The present article planned to study clinical signs, pathological description and the molecular identification of SPPV with PCR.Phylogenetic analysis based on sequencing ofthe PCR products was also performed using genome sequence data from capripoxvirus isolates from all over the world.

MATERIALS AND METHODS

Animals: A sheep farm at Al Hassa District was struck by the sheep pox virus during winter 2014.The farm has not been vaccinated and there is also history of new entrance of animals.The clinical signs were a sudden onset of fever, which peaks at 40–42°C, with discharges from the nose and eyes, depression and excessive salivation.The animal loses its appetite and is reluctant to move.Mortality rate and mortality rate was up to 60% and 90% respectively.

Necropsy and samples: Necropsy examination was carried out on dead animals. Tissue samples from skin lesions of different parts as well as from the lungs were fixed in 10% neutral buffered formalin and processed routinely. Paraffin wax-embedded sections were stained with haematoxylin and eosin (HE). Similar tissues werealso collected, transferred to the Central Biotechnology Laboratory at the College of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia, and stored at -80ºC until used.

Molecular detection of sheep pox virus: According to manufacturer`s instruction, DNA was extracted from skin and lung samples as well as commercial live attenuated sheep pox virus as positive control using DNeasy Blood and Tissue Kit (QIAGEN, USA). DNAs were subjected to multiplex PCR assay to diagnose sheep pox virus infection using KS-1.5 5′-GTGTGACTTTCCTGCCGAAT-3′, KS-1.6 5′-TCTATTTTATTTCGTATATC-3′, InS-1.1 5′-AGAAACGAGGTCTCGAAGCA -3′ and InS-1.1′5′-GGAGGTTGCTGGAAATGTGT -3′ primers (Mangana-Vougiouka *et al.,* 2000). Thermal cycling parameters were initial denaturation at 95ºC for 5 minutes then 35 cycles of 94ºC for 30 second, 43ºC for 30 second and 72 ºC for 30 second and a final extension step at 72 ºC for 10 minutes. PCR products were electrophoresed in 1.5% agarose gel stained with ethidium bromide and documented using ultraviolet gel documentation system (BIORAD).

Sequencing and phylogenetic tree analysis*:* P32 gene (envelope protein) was amplified using B68 5′-CTAAAATTAGAGAGCTATACTTCTT-3′and B695′-CGATTTCCATAAACTAAAGTG-3′ primers (Heine *et al.,* 1999). The thermocycling conditions as in duplex PCR except the annealing temperature was 48 ºC. 390bp PCR specific band was sequenced in an automated ABI 3730 DNA sequencer (Applied Biosystems, USA). The obtained sequence was analyzed using online BLAST server and compared with capripoxviruses sequences available in GenBank (Table 1). A phylogenetic tree was constructed using MEGA version 5.2 software.

GenBank accession number: The obtained P32 gene sequence of detecting SPPV during 2014 was submitted to the GenBank database with the accession number (KP342531), Sheeppox virus strain SPPV/Al-Hassa/2014/KSA.

RESULTS

Necropsy findings: All of the necropsied sheep had numerous skin lesions included erythematous macules and papules, measured 2 to 4 mm in diameter, which may become larger and nodular with typical demarcation. When the necrotic centers of the nodules were removed, the lesions appeared ulcerated. Pustular changes were also observed.The lesions were found all over the body, but were most prominent on the face, back and tail (Fig. 1, a, b, c). The lungs contained areas of congestion, oedema and consolidation, and often had multiple firm white nodules(2-15 mm diameter) disseminated throughout the lobes (Fig. 1d). These nodules were red and surrounded by a zone of hyperemia.

Microscopic findings: The epidermal layers showed thickened, hyperplastic epithelium with hydropic degeneration, particularly in stratum spinosum. Intracytoplasmic eosinophilic inclusion bodies were visible (Fig. 2a). The dermal layer revealed inflammatory exudate characterized by fibrin and cellular debris and massive number of neutrophils (Fig. 2b). Other mononuclear cells consisting of lymphocytes, plasma cells, and some macrophages were observed (Fig. 2c). The blood vessels were congested and showed perivascular cuffs with lymphocytes and macrophages. In the lung, epithelial hyperplasia with squqmous metaplasia were noticed in the terminal bronchioles. In some areas of lung, alveolar walls were thickened due to hyperplasia of type II pneumocytes (Fig. 2d). In most cases alveoli were filled with inflammatory exudate consisting of fibrin, macrophages and lymphocyte

Molecular detection and sequencing of SPPV: Suspicious papule and nodule samples were positive in duplex and P32 PCR test. Amplicons of approximately 149 and 289 bp and 390 bpwere obtained in both PCR assays, respectively (Fig.3). Partial open reading frame (ORF) of P32 gene was sequenced and analyzed using the online BLAST server. The result of BLAST analysis showed that the Sheeppox virus strain SPPV/Al-Hassa/2014/KSA was very close relationships with other *Capripoxviruses* isolated worldwide with nucleotide sequence identity from 95 to 100%. The nucleotide sequence identities were 99 ~ 100%; 95 ~ 98% and 96 ~ 98% with SPPV, GTPV and LSDV, respectively.

Phylogenetic analysis: Phylogenetic tree was performed using MEGA program version 5.2 (Fig.4). Analysis of the phylogenetic tree showed that the strain (SPPV/Al-Hassa/2014/KSA) was clustered with (SPPV/Al-Hassa/2013/Saudi Arabia) and closely related to SPPV strains identified in China and India.

Table 1. *Capripoxviruses* used in the phylogenetic tree construction.

Virus name	Country	GenBank Accession
SPPV/Al-Hassa/2013/Saudi Arabia	Saudi Arabia	KF204447
SPPV/Rumanian Fanar/India	India	AY368684
SPPV/Rainpet/India	India	DQ431989
SPPV/Makhdoom-2007/India	India	EU314721
SPPV/Pune-08/India	India	FJ882029
SPPV/Shanxi/China	China	HM770955
SPPV/Maha/sheep/22/India	India	KF468761
SPPV/GanSuGT/11/2012/China	China	KF661974
SPPV/GanSuHN/12/2012/China	China	KF661977
SPPV/Jilin/China	China	KF991005
SPPV/Kurdistan/Iraq	Iraq	KF992798
SPPV/Lx/Gs/China	China	KJ026555
SPPV/Anyang/Henan/China	China	KJ026553
LSDV/2/Slemani/Kurdistan/2014/Iraq	Iraq	KM047051
LSDV/9/Slemani/Kurdistan2014/Iraq	Iraq	KM047058
LSDV/Slemani/Kurdistan/2013/Iraq	Iraq	KF996498
GPPV/Yemen (Sanaa/1983)/Yemen	Yemen	EU625262
GPPV/Maha/goat/19/India	India	KF468762
GPPV/JK/sheep/27/India	India	KF468758
GPPV/ChongQ/2009/China	China	HM572329
GPPV/Viet Nam (NinhThuan/2005) / Viet Nam	Viet Nam	EU625263
GPPV/JK/goat/27/India	India	KF468757
LSDV/Neethling vaccine LW 1959/South Africa	South Africa	AF409138
LSDV/Neethling	South Africa	AF336131
LSDV/Neethling 2490	Kenya	AF325528
LSDV/NeethlingWarmbaths NW-LW	South Africa	AF409137
GPPV/G20-LKV	Kazakhstan	AY077836
GPPV/Pellor	Kazakhstan	AY077835
SPPV/NISKHI	Kazakhstan	AY077834
SPPV/ strain A	Kazakhstan	AY077833
SPPV/TU-V02127	Turkey	AY077832
SPPV/Nigeria 1	Nigeria	AF124517
SPPV/RM/65/Iran	Iran	FJ917518

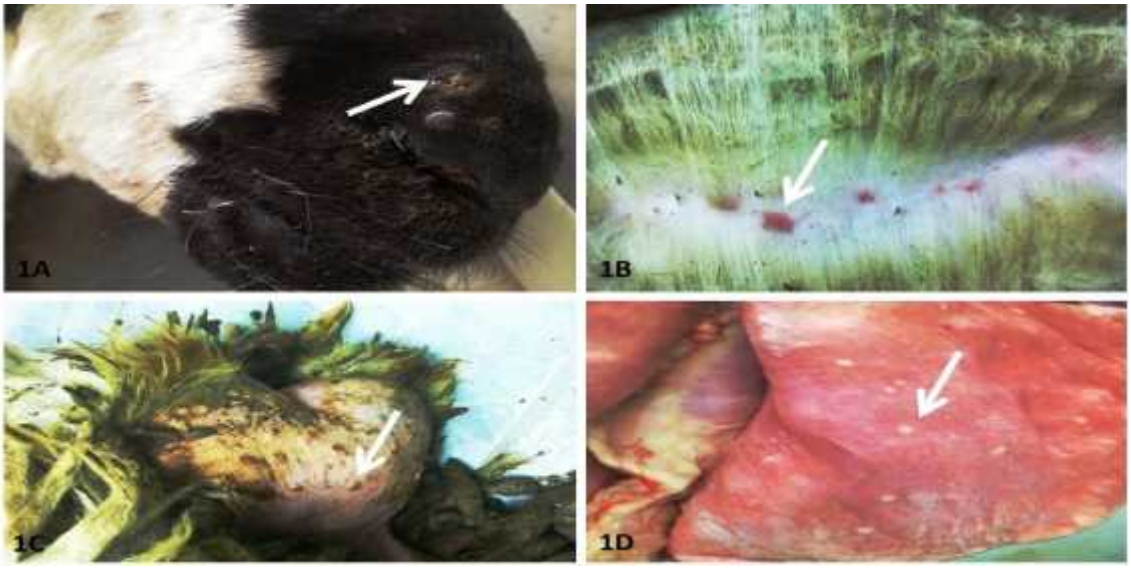

Fig. 1. Sheep pock lesions. A- Pock ulcer (Nose). B- Pock erythema (skin). C- Pock papule (tail). D- Pock nodules (lung).

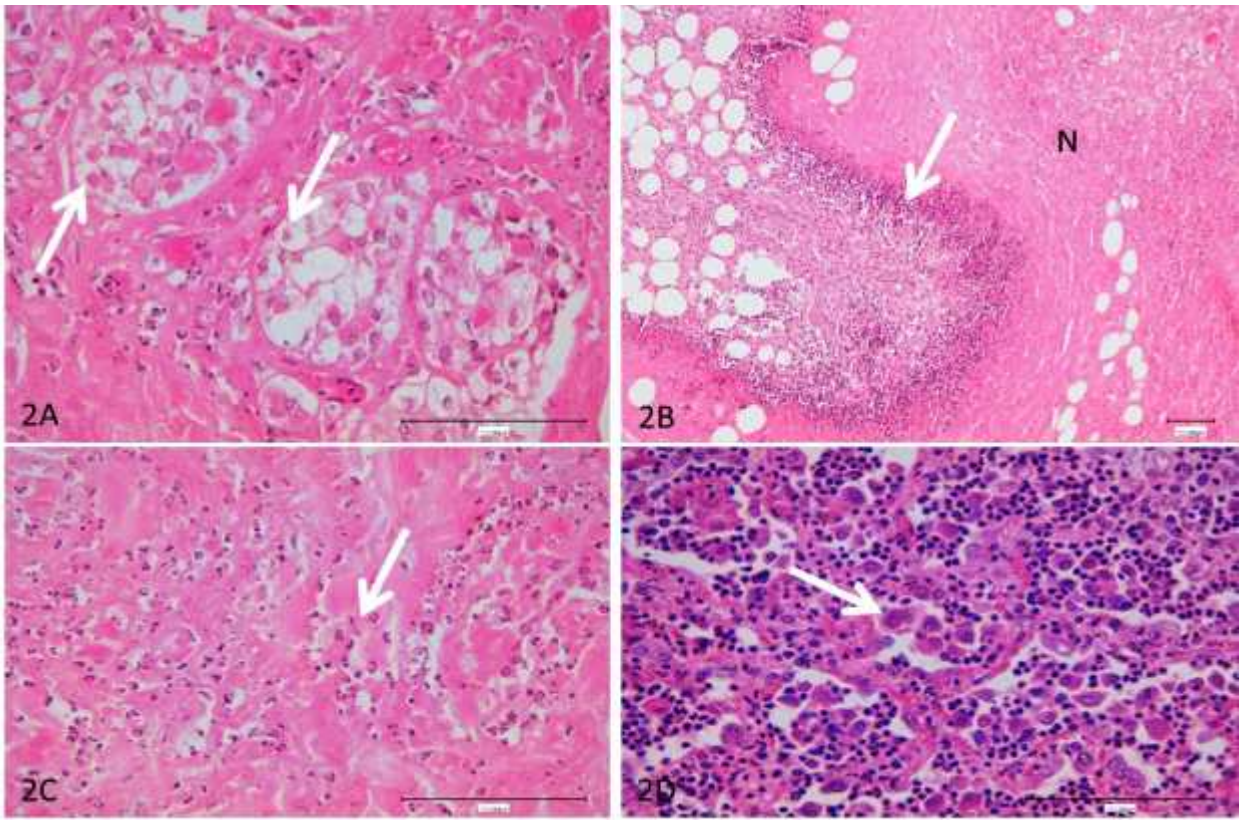

Fig. 2. A- Eosinophilic intracytoplasmic inclusions in stratum spinosum (arrows). H&E, scale bar = 100 µm. B- Area of necrosis (N) surrounding with a zone of inflammatory cells (arrow). H&E, scale bar =100 µm. C- Pustule, heavy neutrophils infiltration (arrow). H&E, scale bar = 100 µm. D- Pneumonitis, hyperplasia of pneumoyte type II (arrows). H&E, scale bar = 100 µm.

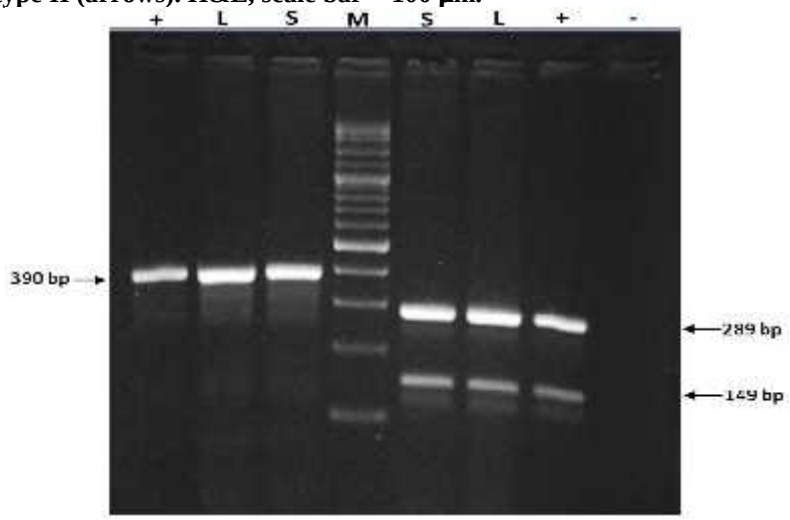

Fig. 3. Agarose gel electrophoresis of the PCR products of the P32 gene (390 bp) and duplex PCR (149 and 289 bp). Lane M, molecular weight marker; Lane S, represents a skin sample; Lane L, represent a lung sample; Lane +, Sheeppox virus vaccine served as positive control and Lane -, Water served as negative control.

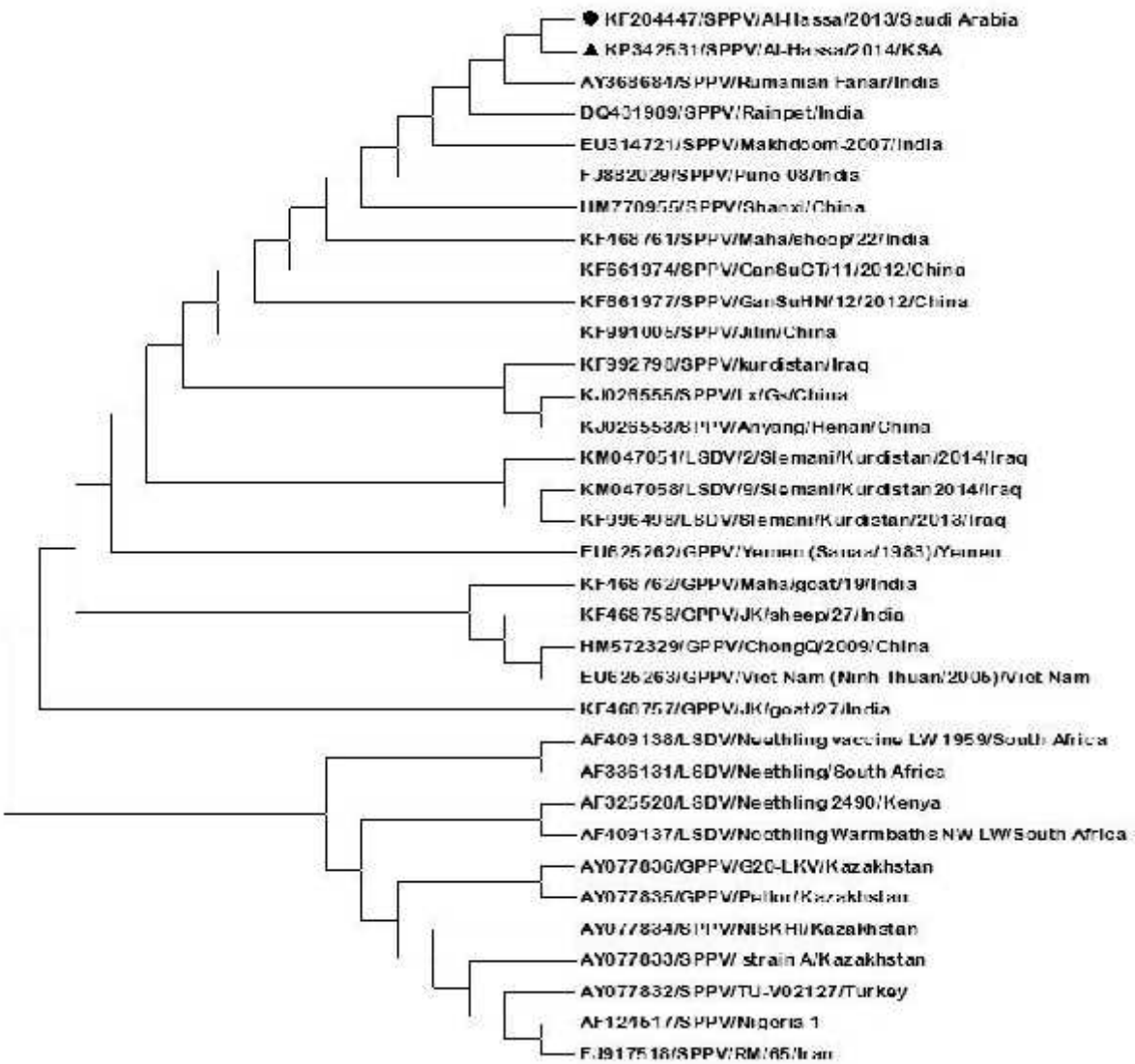

Fig. 4. Phylogenetic tree of *Capripoxviruses* based on partial nucleotide sequences of P32 gene. The Saudi Arabia strains are marked with solid circle and triangle for 2013 and 2014 strains, respectively.

DISCUSSION

Capripoxviruses have the potential to become an emerging and bioterrorism agents because of worldwide climate change, an alteration in trade and animal products (Babiuk *et al.,* 2008). So far, the current situation of sheep pox infection among sheep and goats in Saudi Arabia still needs more investigation about the varieties of strains, the economic losses and the definitive diagnosis.These attributes increase the prospect of successfully implementing country control program. During winter, 2014 an outbreak of the disease strucka sheep farm which is not previously vaccinated and also has a history of new entrance of animlas. Previous studies stated that varying degree of disease might be due to the influence of host, agent, environmental conditions, the increase of illegal animal movement through trade and husbandry practices (Eroksuz *et al.,* 2008). Clinical signs of illness were fever and respiratory signs, Mortality rate reach to 60 %, while mortality rate was up to 90% and mostly confined in lambs (Bhanuprakash *et al.,* (2005) and Sharma *et al.,* (2013).There is a consistent specific pox skin lesion in all infected animals and the lesions were frequently in the form of macules and papules, and obviously seen in unwooled areas in the head, abdomen and tail (Babiuk *et al.,* 2008 and Bhanuprakash *et al.,* 2010).At necropsy, Lung was the only organ affected and there was multiple nodules distributed throughout lung lobes, and this findingwas considered a consistent necropsy findings in all dead lambs (Chani, 2011). Histopathological examination of skin lesion in all infected sheep showed that the epidermal and dermal

changes associated with hyperplasia of pneumocyte type IIand the presence of intracytoplasmic inclusions were considered a characteristic lesion of the disease in sheep (Singh *et al.,* (2007); Zangana and Abdullah, (2013)). Furthermore, the molecular diagnosis of disease which based on alignment and phylogenetic analysis of P32 gene showed that that there is a close relationship among Saudi Arabia SPPVs detected during 2013 and 2014 as they are clustered together in a separate sub-cluster (Abdulkarem *et al.,* 2014).Additionally, the Saudi Arabian SPPVs detected during 2013 and 2014 were closely related to Indian and Chinese SPPVs.The occurrence of this outbreak together with the previous outbreak in 2013 highlights the fact that sheep pox is an emerging pathogen in this District and an affect susceptible host during stress weather. Therefore, it is important that the state animal husbandry along with other stakeholders keep strict vigil on the development of disease among animals in susceptible areas.Also, quarantine of new animals before introduction to the herd and immediate implementation of mass vaccination, slaughter and proper disposal of affected animals is very important to prevent any further losses of disease.

Acknowledgements: The authors are thankful to the Deanship of Scientific Research, King Faisal University, Saudi Arabia for support this work with a financial grant number: 150048

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