A lumpy skin disease case in the southeast turkey: A threat for Eurasia

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ABSTRACT
Lumpy skin disease (LSD) is a pox disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. A disease form was observed in cattle in the Southeast Anatolia in 2013 and 2014 and suspected for LSD suspected by clinical examination. In this study, LSD has been defined by molecular virologic and histopathologic tests. A necropsy was undertaken and tissue samples were taken. Also, blood and tissue samples with biopsy punches were taken from animals having symptoms. Tissue and blood samples were examined molecularly and histopathologically while tissue samples were examined histopathologically. Positive amplification was detected by Polymerase Chain Reaction and confirmed by sequencing in the extracted DNA’s derived from tissues. Tissue samples were compatible with LSD histopathologically. The first official notification of LSD in Sanliurfa province of the Southeast of Turkey is determined. As a result, LSD could be treated northern territories in suitable climate conditions.

Key words: Histopathology, Lumpy skin disease, PCR, Sequencing

INTRODUCTION
Lumpy skin disease (LSD) is a pox disease of cattle (OIE, 2014a; EFSA, 2015). Infection causes huge economical losses and effects international trade (Davies, 1991; EFSA, 2015; Ince et al., 2016). The causative agent is a pox virus that is called by the same name, Lumpy skin disease virus. LSD virus belongs to the genus Capripoxvirus within the family Poxviridae (Buller et al., 2005). The prototype of LSD virus is Neethling strain (Alexander et al., 1957). It is agreed that Capripoxviruses are considered to be host specific (Babiuk et al., 2008).

The virus causes an acute or subacute systemic disease table in cattle (Davies, 1991). Disease is characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death (Davies, 1991; Knowles, 2011; OIE, 2014a). According to international and national regulations, it is a notifiable disease (Anonymous, 1989; Anonymous, 2011; OIE, 2014b). Transmission of LSD virus is thought to be mainly mechanically by insects (Carn and Kitching, 1995; Chihota et al., 2001; OIE, 2014a). LSD virus is not transmissible to humans (OIE, 2014a).

LSD was first recorded in 1929 in Zambia. Up to the year 1950, it was observed in Botswana, South Africa and Kenya. Since then, its distribution has extended throughout the sub-Saharan and Western African countries (Sudan, Nigeria, Mauritania, Mali, Ghana, Liberia, Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon) (OIE, 2014a). LSD is endemic in many African countries and it is suddenly spreading the Middle East countries in the years 2000s (Tuppurainen and Oura, 2012; Wainwright et al., 2013; Tuppurainen et al., 2014; EFSA, 2015). Disease is still spreading northward (Tuppurainen and Oura, 2012; Tuppurainen et al., 2014). LSD was first reported to The World Organisation for Animal Health (OIE) in 2013 in Turkey (Sarac et al., 2014; Anonymous, 2013).

The diagnosis of LSD can be made based on characteristic clinical signs. However, laboratory methods are needed to confirm the presumptive diagnosis (OIE, 2014a; EFSA, 2015). Histopathological, virological, molecular and serological methods can be used for this purpose as directly or indirectly. Blood, serum, semen, tissue culture and tissue samples may be used as a starting diagnostic material (OIE, 2014a). The Capripoxvirus genus shares a common major antigen for neutralising antibodies and it is not possible to distinguish strains using serological techniques. Therefore, it requires extra tests for the differential diagnosis (Manimaran et al., 2017; OIE, 2014a). Laboratory confirmation of LSD is most rapid using a polymerase chain reaction (PCR) method in recent years (OIE, 2014a). Also, it is expressed that the PCR-based test

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for *Capripoxviruses* has better analytical and diagnostic sensitivity than the other tests (Carn and Kitching, 1995). But, it is suggested that strains of virus should be identified by the use of sequence and phylogenetic analysis, because of the detected genes shared by all *Capripoxviruses* (Le Goff et al., 2009; OIE, 2014a).

LSD has not been notified in Turkey until 2013 in accordance with accessible literature. The quite lot number of farmers from Sanliurfa and surrounding provinces have applied and wanted special recipe and treatment for a disease clinically similar to LSD in 2003 and 2014. Unfortunately, we had not taken diagnostic samples from animals belonging to these farmers. Because, the farmers had hesitated from quarantine measures in the case of the official disease notification in accordance with current animal health rules. Also, authorized local official veterinary authorities had also ignored in this situation.

The aim of this study was to diagnose an unknown animal disease and to report to the official authorities and to describe identity of the etiological agent by molecular tests and histopathologic examination in cattle in Sanliurfa and surrounding provinces.

**MATERIALS AND METHODS**

**The disease outbreaks:** This study was carried out in the Sanliurfa province of Turkey (located in 37° 10’ 2.6544’’ N and 38° 47’ 43.8468’’ E). Disease outbreaks occurred in the local farms housing different breeds of cattle in 2013 and 2014 in Sanliurfa and surrounding provinces. The disease resembled in looks clinically similar to LSD as can be seen in Figure 1, the affected cattle showed clinical signs of sickness and lesions on the skin. Finally, the governmental farm was requested an investigation in May 2014 in Sanliurfa. Disease investigation was undertaken and necropsy was performed on selected one Holstein breed of adult cattle in the farm. Samples collected for laboratory investigation included scabs, blood, pieces of internal organs and lymph nodes (ear number is 361). Also blood and biopsy specimens were taken from the other 3 affected animals (ear numbers are 227, 236, 81) showing high body temperature. The supportive treatment and quarantine measure were recommended.

**DNA extraction:** Firstly, all blood and skin biopsy samples were homogenized (10% weight/volume in Dulbecco’s Modified Eagle’s medium, D-MEM) in 2.0 ml tubes (Triple-Pure High Impact Zirconium Beads, Ø: 3.0mm) using Bead Bug Microtube Homogenizer (Benchmark Scientific, USA). The DNA from samples was extracted with the Gene JET Genomic DNA Purification Kit (Cat. No. K0721, Thermo Scientific, Germany), according to the manufacturer’s instructions and eluted in 50µL of elution buffer included in the same kit. The DNA concentrations were measured with UV visible spectrophotometer (DeNovix, DS-11, USA) and diluted as 100 µg/µl.

**Polymerase Chain Reaction:** The PCR primers (forward primer 5'-TTTCCGTATTTTCTTACTAT-3'; reverse primer 5'-AAATTATATACGTAAATAAC -3') were developed from the viral attachment protein encoding gene and the size of the amplicon was 192 bp (Ireland and Binepal, 1998). One-step RT-PCR kit (Qiagen, Germany) was used for amplification of nucleic acid templates. DNA amplification was carried out in a final volume of 25 µl containing 5 µl5X RT-PCR Buffer, 5 µl 5X Q-Solution, 1 µl
dNTP Mix, 1 µl Enzyme Mix, 1 µl 0.20 mM each primer, 10 µl distilled water and 1 µl DNA sample. The reaction was carried out in a Hybaid PX2 thermal cycler (Thermo Fisher Scientific, USA). Thermal cycling proceeded at 95 °C for 3 minutes, followed by 34 cycles of 95 °C for 45 seconds, 46 °C for 30 seconds and 72 °C for 1 minute and a final elongation step 72 °C for 5 minutes (Ireland and Binepal, 1998). Amplified products were analysed using a 100 bp DNA ladder (GeneRuler™ 100 bp DNA ladder, Fermentas, Thermo Fisher Scientific, USA) as a molecular marker on 1.5% agarose gels. Gels were stained using ethidium bromide (1 µg/ml) in tris borate EDTA (TBE, 10x liquid contains 1.3M TRIS, 450 mM boric acid and 25 mM EDTA in water) and amplicons were visualized using an UV transilluminator.

A water control was used as a negative in all steps. The resulting DNA fragments were visualised by ultraviolet transillumination and photographed. The visible bands of the appropriate size (192 bp) were considered a positive reaction and were confirmed in the national reference laboratory (The Reference Laboratory of Diagnosis Sheeppox and Goatpox, Istanbul Pendik Veterinary Control Institute, NRL).

**Histopathology:** Firstly, samples (taken from biopsy and internal tissues) were treated with 10% formaldehyde. After that, fixed tissues were incubated in the serial alcohol and xylol tanks. Incubated tissues were blocked with paraffin. Paraffinized blocks were cut 4-6 µm and stained with hematoksielen-eozin (HE). Stained glasses were checked under the light microscope.

**Sequencing:** The P32 gene of LSD viruses from the outbreak was sequenced partially and compared with archived viruses from GenBank. Sequence applications were done commercially using same PCR primers. The sequences were aligned and edited using Bioedit software (Hall, 1999).

**RESULTS AND DISCUSSION**

The suspected disease form which clinically diagnosed as LSD was confirmed in the laboratory by molecular-virological and histopathological tests. Also, this LSD diagnosis was confirmed by NRL of Turkey. This confirmation was checked using DNA sequencing.

**Histopathology:** Histopathological section of all samples (skin biopsy, tissues taken from autopsy) shows changes peculiar to LSD. The vasculitis and perivascular infiltration with white cells were seen in light microscope. There were eosinophilic intracytoplasmic inclusions in the lesions and the inflammatory cells as can be seen in Figure 2. The lesions seemed necrotic as a result of the thrombosis.

**PCR:** Three out of four (75%) tissue specimens were positive, while 0 of four (0%) blood specimens were positive with PCR, as shown in Figure 3. Disease notification was announced to the Sanliurfa Provincial Directorate of The Ministry of Food Agriculture and Livestock.

**Sequencing:** The P32 gene of three PCR positive field isolates (361, 227, 236) was sequenced partially. The very short sequences of P32 gene were obtained (151-174 bp). Neethling Strain sequence (AF325528) was obtained from GenBank and used for alignment and comparison (Tulman et al., 2001). Alignment and comparison of sequencing results with a Neethling Strain indicated 100% compliance and the circulating virus was LSD virus, as can be seen in Figure 4. Therefore, sequencing results of the positive PCR amplifications confirmed that all three virus isolates from outbreak were LSD virus.

In the submitted study, LSD was confirmed by molecular-virological and histopathological tests in the Southeast Anatolia. Also, it was the first report of the LSD case in Sanliurfa province. Furthermore, the study was one
or two attempt in the Turkey which molecular-virological and histopathological tests were used at the same time.

It is argued that rapid laboratory confirmation of poxvirus diseases based on clinical signs, electronmicroscopy and serological tests are not always reliable. Also, virus isolation in cell culture and Enzyme Linked Immuno Sorbent Assay (ELISA) fail to detect virus particles because of neutralizing antibody (Ireland and Binepal, 1998). For these limitations, PCR techniques have been advised to detect poxviral DNA in infected cell culture supernatants, biopsy samples (Bhanuprakash et al., 2006). On the other hand, OIE and European Food Safety Authority (EFSA) have suggested special tests that they could be rapid and implacable in the field for the diagnosis of LSD (OIE, 2014a; EFSA, 2015). In the last decade, it is reported that PCR have been used very extensively for the diagnosis of LSD (Tuppurainen et al., 2005; OIE, 2014a; EFSA, 2015). Also, histopathology and immunohistological staining provide a relatively inexpensive tool to diagnose the disease (Tuppurainen et al., 2005).

Analytical sensitivity of PCR based tests using biopsy specimens is higher than the other tests such as ELISA for Capripoxviruses (Tuppurainen et al., 2005). Also, rapid diagnosis of disease is so important for implementing prevention and control measures quickly, because of its vector borne nature. From this point, histopathologic and molecular virological methods are preferred in this study. Another factor is having suitable laboratory infrastructure and preparedness for LSD outbreaks.

The diagnosis of LSD in Turkey using histopathologic and molecular virological methods was reported by Uyar et al. (2015). Histopathology and real time PCR tests were used in this study using tissue and blood specimens. Researcher detected viral nucleic acid of LSD.
virus from blood specimens. But none of the blood sample was positive in present study. The results of studies are incompatible for blood specimens. The nucleic acid isolation method used in our study was probably not compatible in terms of the DNA obtained from the blood material. Histopathologic finding were similar in both studies. Uyar et al. (2015) had not used confirmatory test such as sequencing after PCR which was advised by OIE and Le Goff et al. (Le Goff et al., 2009; OIE, 2014a). We used sequencing and phylogenetic analyses for confirmation, it seems to be important. Because, it is reported that primers for the viral attachment and the fusion protein genes are specific for all the strains within the genus *Capripoxivirus*. Strains of virus can be identified by the use of sequence and phylogenetic analysis (Le Goff et al., 2009; OIE, 2014a). Therefore, our study is considered to be in full compliance with the OIE in terms of methodology.

The first LSD outbreak in Turkey was announced by Sarac et al. internationally in 2014 (Saraç et al., 2014). It is reported that biopsy, blood and nasal discharge specimens of 8 different animals were used in these study and positive amplifications were detected in 4 of biopsy, 3 of blood and 1 of nasal discharge specimens with PCR. Also, researcher used confirmatory test after PCR detection. They had not used histopathologic test. Authors said that source of the outbreaks in Turkey were mainly associated with global climate changes and uncontrolled animal movement. Our PCR findings and predictions were similar for LSD outbreaks.

In another study, PCR was also used for diagnosis of LSD in Turkey (Gürçay et al., 2015), authors tested 12 biopsy specimens taken from different cases against LSD virus. They found positive amplification in all the specimens. In the near geographical region of Turkey, Sharawi and Abd El Rahim (2011) used PCR to diagnose of LSD in Egypt. Researcher tested 10 different biopsy specimens and detected positive amplification in all the samples (Sharawi and Abd El Rahim, 2011).

The most comprehensive studies were conducted by Işcan et al. (2014) and Sevik and Dogan (2016) in Turkey. Researchers used molecular tests and phylogenetic analysis tools. Işcan et al. (2014) developed a spreading model using geographical information system (GIS). It seems to be useful tool for the Ministry of Food, Agriculture and Livestock. This report clearly indicated that the disease is spreading northward in Turkey and their territories. Sevik and Dogan (2016) analysed molecular and epidemiological data in a large area of Turkey, also advised control strategies and risk analyses.

There are 7 provinces in the South borderline of Turkey (Hatay, Kilis, Gaziantep, Sanliurfa, Mardin, Sanliurfa and Hakkari). But the first LSD case in Turkey was reported from Elbistan/Kahramanmaras and Gercü/Batman (Anonymous, 2013; Saraç et al., 2014), which are quite far away from borderline. Also LSD was seen in Sivas province in the Central Anatolia before the border provinces (Anonymous, 2014). The timeline of chronological spreading of diseases in Turkey can be found from web pages of the ministry and OIE-WAHIS (Anonymous, 2013; Anonymous, 2014). This situation was not familiar for transboundary diseases. When evaluated in terms of disease prevention and epidemiological control methods this should be considered as an important problem that needs more attention.

United Nation, Food and Agriculture Organization (FAO) said that LSD probably travelled to Turkey from Syrian Arab Republic because of the country’s current armed conflict. Also FAO has warned about that the disease may continue spreading north, west and east from Turkey. FAO indicated that there are different reasons (uncontrolled livestock movements, seasonal transhumance movements, suitable riverine agriculture for vector transmission and global climate change) for spreading of LSD in Turkey (Wainwright et al., 2013). We share this determination and would like to add some observation. Firstly, all notifications have been ignored about the possible spreading of disease from the Middle East and measures have not been taken in Turkey. Therefore, the Ministry of Food, Agriculture and Livestock should set up a computer based early warning alert system, epidemiologically. The implementation of animal health act in the borderline provinces of Turkey should be monitored strictly and adaptation should be announced according to the national and international commercial concerns. Another important factor is the meteorological events for vector-borne diseases like LSD. It should be noted that Turkey (also the Middle East) is constantly under the influence of repeated wind systems (Braverman and Chechik, 1996). Meteorological events (wind systems) could be origin of disease in this case as explained by Braverman and Chechik (1996). From time to time, vector-borne diseases have been seen for these effects seasonally in Turkey (bluetongue, akabane, bovine ephemeris fever etc). The disease patterns are affected by global climate change, epidemiologically (Saraç et al., 2014; Wainwright et al., 2013). LSDV may also be used as the economic bioterrorism agents in this case. These possibilities are clearly reported in 2008. Report says that “*Capripoxviruses* have the potential to become emerging disease threats because of global climate change and changes in patterns of trade in animals and animal products. They also could be used as economic bioterrorism agents” (Babiuk et al., 2008).

In summary, clinically LSD suspected cattle were seen in Sanliurfa in 2013-2014. The samples were obtained, tested, sequenced and characterized from these animals. Histopathologic results supported this clinical suspicion. It is clear that the circulating virus is LSD virus according to...
molecular virologic tests. The multiple sequence alignments and phylogenetic analysis results are confirmed this finding. The first official notification of LSD in Sanliurfa province is determined. Also, results suggested that further investigations of the full genome sequencing of the circulating viruses are needed for detailed understanding of outbreak. As a result, LSD could be treated northern territories in suitable climate conditions.

REFERENCES


