

Case Report

Confirmatory diagnosis of contagious ecthyma (Orf) by polymerase chain reaction at Adet Sheep Research Sub-Center, Ethiopia: A case report

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An outbreak of contagious ecthyma (CE) was investigated in June, 2012 with morbidity rate of 22% in Adet Sheep Research Sub-Center, Ethiopia. The results of this investigation indicated that the outbreak was caused by infection with CE virus. A polymerase chain reaction (PCR) assay for rapid diagnosis was applied to five scab samples obtained from sheep suspected for CE. To confirm whether the causative agent was present in skin scrapings, PCR of the complete B2L gene to diagnose CE was used in this study. The expected PCR fragments, approximately 1206 bp in length were obtained from DNA which had been extracted from tissue scrapings. All five skin scab samples were confirmed positive to CE by PCR. In conclusion, detailed phylogenetic analysis of CE virus is suggested in order to know the genetic origin of the virus strain as well as for the future choice for immunoprophylaxis.

Key words: Adet Sheep Research Sub-Center, B2L gene, contagious ecthyma, polymerase chain reaction (PCR), sheep.

INTRODUCTION

Contagious ecthyma (CE) virus is the etiological agent of contagious pustular dermatitis and is the prototype of the genus *Parapox virus* (PPV), which is an oval, enveloped virus containing dsDNA genome within the genus *P. virus*, family Poxviridae (Damon, 2007). This epitheliotrophic virus causes a severe exanthematous dermatitis that afflicts domestic and wild small ruminants (Robinson and Balassu, 1981; Keshan et al., 2010). It is characterized by the formation of papules, nodules, or vesicles that progress into thick crusts or heavy scabs on the skin of the lips, on the oral mucosa, tongue, gingiva,

and around the nostrils (Robinson and Balassu, 1981; Vikoren et al., 2008). Lesions can also be found occasionally on the teats of nursing animals and rarely on other organs (Vikoren et al., 2008). Characteristic of the disease are proliferative and often self-limiting lesions (Keshan et al., 2010).

The morbidity of the disease may reach 100%. The mortality rate related to CE is usually low, but it may be very high in small ruminants, especially when bacterial or fungal secondary infections occur (Robinson, 1983; Lughano and Dominic, 1996; Haig and Mercer, 1998).

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The disease does not only have an economic impact on farmers worldwide, but also have a considerable negative effect on animal welfare. Infected animals are sickly, fail to thrive, and are more susceptible to adventitious bacterial infections (Gallina et al., 2006). CE is an infection of public health significance and humans with immunodeficiency diseases, in particular can develop serious infections (Ara et al., 2008).

Although clinical diagnosis, histopathology and electron microscopy have been used for viral identification, only polymerase chain reaction (PCR) and genomic analyses can distinguish CE virus from other *P. virus* species. The PCR method based on the amplification of the B2L gene technique has become a powerful tool in molecular diagnosis of CE (Inoshima et al., 2000; Inoshima et al., 2001; Tikkanen et al., 2004). The diseases caused by *P. virus ovis* or Orf virus (ORFV) have worldwide distribution and have been reported from many countries (Hosamani et al., 2006). Although, outbreaks of CE have been reported in Ethiopia, confirmatory diagnosis using molecular technique has not been conducted. Thus, the objective of this study was to confirm CE virus from clinical samples by PCR assay by amplifying a part of B2L gene of CE virus.

MATERIALS AND METHODS

Study area description

This case study was conducted at Adet Sheep Research Sub-Center, found in Yilmanadensa district, Amhara Region, Ethiopia, which is located at about 40 km in South-East of Bahir Dar 11°10' to 11°15' N and 37°30' to 37°40' E at an altitude range between 1500 and 3000 m above sea level. It has a uni-modal type of rainfall receiving a mean annual rainfall of about 1270 mm (1051 to 1488 mm) which occurs from May to October. The research sub-center contained 112 Washera sheep in the time of sample collection.

Study animals and flock management

Washera sheep (known as Agew or Dangla sheep) of both sex and all age reared at the research sub-center were the study animals. This sheep breed is characterized by large body size, wide fat-tail usually curved upward tip, horizontally carried or semi-pendulous long ears, both sexes hornless, slightly concave facial profile with plain, patchy and spotted patterns of coat colour (Sisay, 2009).

Grazing constitutes the basal ration throughout the year. Sheep were allowed to graze the natural pasture for 8 to 10 h per day. During dry season where feed scarcity is common, sheep were supplemented with vetch hay, concentrated feed containing maize, noug cake and wheat bran. Pure breeding was performed in the research sub-center with sex ratio of 1 ram to 20 to 30 ewes. The flock was structured into five groups (each group containing 1 ram with 20 to 30 ewes).

Foot rot, CE, gastro-intestinal parasites, especially *Haemonchus contortus* and ticks were the common ovine diseases reported in the research sub-center. Tentative case reports from the research sub-center show that CE was the primarily sheep health constraint. An annual outbreak of CE repeatedly occurred during wet season Vaccination against CE has not been practiced in the research sub-center. Strategic vaccination was given for Ovine Pasteurellosis, Anthrax, and Sheep pox. Sheep were strategically dewormed

with broad-spectrum anthelmintic against gastro intestinal parasites (GIP) according to the area rain fall pattern; at the beginning of rainy season (June), at mid dry season (March to April) and at the end of rainy season (October). Scheduled dipping and spraying against to external parasite infestation were also conducted.

Case presentation

At the beginning of wet season, June 2012, a high incidence of disease showing skin lesions in the form of papules, pustule, ulcers and brown thick scabs on the lips, gingival, knee and inter-digital region was observed among sheep in Adet Sheep Research Sub-Center.

For specimen collection, five (5) sheep were presented with skin lesions in the form of pustule, ulcers and brown thick scabs on the lips, gingival and wart-like lesions on the feet (knee and interdigital region), and ear (Figure 1A to F).

Specimen collection and laboratory technique

Skin scrapings were collected from five affected sheep and were stored frozen until testing. Then collected samples were dispatched to National Veterinary Institute (NVI), Debre Zeit for laboratory examination. PCR to amplify B2L gene of CE virus was applied.

DNA extraction and PCR assay

The collected scab samples were minced using sterile scissors and forceps and then triturated in a sterile pestle and mortar with phosphate buffered saline and made into 10% suspension. The mixture was clarified at 1800 rpm for 10 min and then transferred to a microcentrifuge tube and subjected to DNA extraction. The DNA was extracted from scab materials by using the phenol: chloroform:iso-amyl alcohol method as described by Klein (2004) with slight modifications. 200 µl of tissue samples were transferred into a 1.5 ml microcentrifuge tube; 200 µl PureLink™ genomic digestion buffer were added and mixed well; 20 µl proteinase K was added and gently mixed, and then incubated at 55°C water bath for 1 h; 20 µl RNase A was added and incubated at room temperature for 2 min, then the lysate was centrifuged at maximum speed for 5 min at room temperature to remove any particulate material; the supernatant was transferred to a fresh microcentrifuge tube; 200 µl PureLink™ genomic binding buffer was added and mixed well by vortexing; 200 µl 96 to 100% ethanol was added to the lysate and mixed well by vortexing for 5 s. Binding of DNA was made by removing the PureLink™ spin column in a collection tube, then lysate of 640 µl was added to the PureLink™ spin column. The column was centrifuged at 10,000 ×g for 1 min at room temperature. The collection tube was discarded and the spin column was placed into a clean PureLink™ collectin tube. Washing of DNA was also carried out by adding 500 µl of washed buffer 1 to the column; centrifuged the column at room temperature at 10,000 ×g for 1 min. The collection tube was discarded and the spin column was placed into a clean PureLink™ spin collection tube, then 500 µl washed buffer 2 was added. The columns were centrifuged at maximum speed for 3 min at room temperature and discard the collection tube. Eluting of DNA was carried out by placing the spin column in a sterile 1.5 ml microcentrifuge tube; 50 µl of PureLink™ genomic elution buffer was added to the column then incubated at room temperature for 5 min; the column was centrifuged at maximum speed for 1 min at room temperature. Finally, the purified DNA was stored at -20°C until analysis.

The extracted DNA was subjected to PCR technique as per the procedure standardized in this laboratory. CE virus specific primers B2L gene, was used in the PCR assay. The primer sequences used

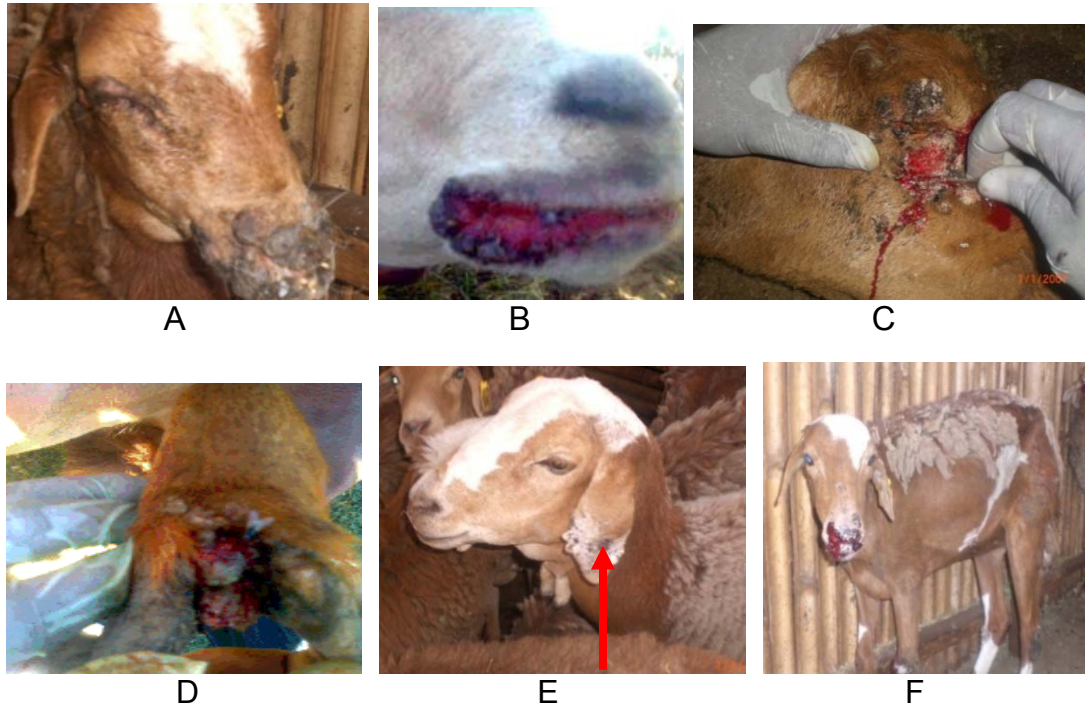


Figure 1. Observed clinical cases of CE virus infection in affected sheep flock at Adet Sheep Research Sub-Center. A, B and F: sheep with severe proliferative scabby and ulcerated lesions of ecthyma around the lips. C and E: wart-like multiple nodules around eyelids and ears. D: wart-like tissue projections in the interdigital space.

for amplification of B2L gene were: Forward primer: 5'-GCT CTA GGA AAG ATG GCG TG-3' Reverse primer: 5'-GTA CTC CTG GCT GAA GAG CG-3'.

The PCR mixture was prepared in total volume of 20 μ l containing DNA template 3 μ l, forward primer 2 μ l, reverse primer 2 μ l, IQ supermix 10 μ l, and distilled water 3 μ l and then subjected to the following PCR cycling conditions in a thermal cycler (Applied Biosystem). Initial denaturation was at 94°C for 5 min followed by 30 cycles denaturation at 94°C for 60 s, annealing at 57°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 7 min. The amplified product was electrophoresis on 2% agarose gel, stained with ethidium bromide.

RESULTS AND DISCUSSION

During clinical examination and sample collection, the clinical picture was quite characteristics; the observable clinical signs were proliferative papular lesions in oral commissures, muzzle and lower jaw regions and tumor-like tissue projections in the interdigital space (Figure 1). The clinical diagnosis performed at Adet Sheep Research Sub-Center suggested CE with morbidity rate of 22% (25/112).

All five clinical samples collected from the outbreak were confirmed as positive with PCR, a product size around 1206 bp (Figure 2). The PCR result of five suspected samples was equal to the PCR reading of positive control CE virus which showed 1206 bp (Figure 2).

The traditional methods of diagnosis that depend on

pathologic examinations and clinical signs are inaccurate, virus isolation is thought to be a gold standard, but it is time-consuming (Chan et al., 2007). But with the development of molecular biology, the PCR technique is being widely used to amplify the desired genomic fragments from tissue specimens of affected animals, and it has become a powerful tool in molecular diagnosis (Inoshima et al., 2000). Thus, to confirm whether the causative agent was present in skin scrapings, PCR of the complete B2L gene was used in this study. The expected PCR fragments, approximately 1206 bp in length, were obtained from DNA which had been extracted from tissue scrapings.

Apart from the present study, different authors have used PCR for the detection CE virus and they have reported that PCR assay is a rapid and specific detection system for the diagnosis of CE (Inoshima et al., 2000; Kanou et al., 2005; Klein and Tryland, 2005; Hosamani et al., 2007; Ramesh et al., 2008).

A conventional PCR method that is based on the amplification of complete sequence of the B2L gene has been used for the detection and phylogenetic analysis of ORFV (Inoshima et al., 2001; Guo et al., 2003; Tikkanen et al., 2004; Guo et al., 2004; Hosmani et al., 2006). Hence, the PCR assay using B2L gene primers proved to be a rapid detection system for the diagnosis of CE for field outbreaks without using other diagnostic techniques like cell culture system or electron microscopy.

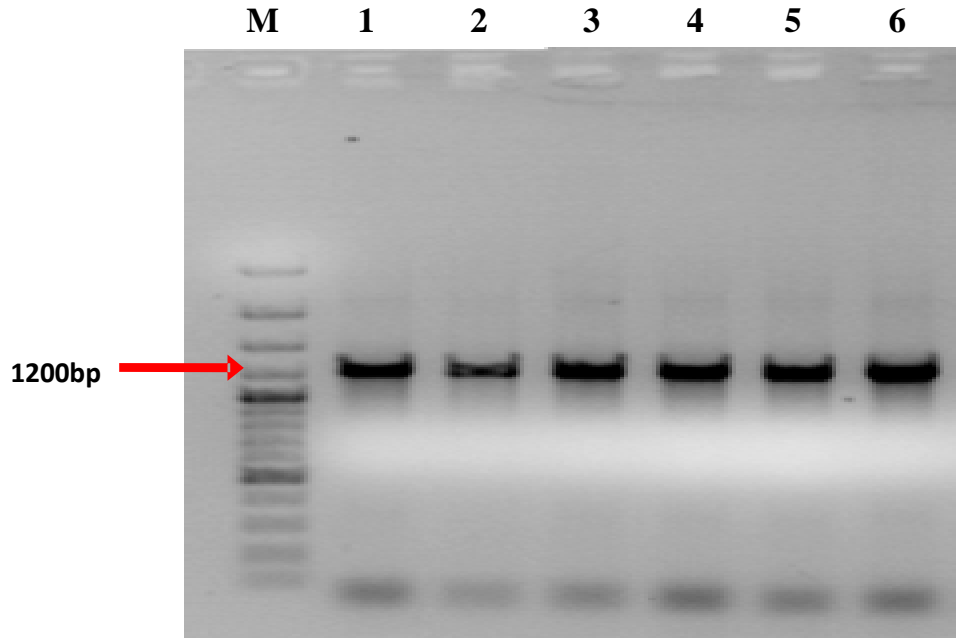


Figure 2. Agarose (2%) gel electrophoresis of major envelope (B2L) gene fragment obtained by PCR, stained with ethidium bromide. M: 100 bp Marker; Lane 1,2,3,4 and 5: PCR amplicon from contagious ecthyma suspected scab samples; Lane 6: PCR amplicon from known contagious ecthyma positive control (1206bp).

The morbidity rate associated with CE in this study was 22% which is lower than the report made by Lughano and Dominic (1996), in sub-Saharan Africa with morbidity rate of 70 to 90% in small ruminants and still high (80%) morbidity rate was also recorded in an unvaccinated flock (Animal Disease Fact Sheet, 2007). The discrepancy of the magnitude of morbidity among previous and current reports may be attributed due to differences in stage of outbreak investigation and patient animal management practices. From Adet Sheep Research sub-Center, CE outbreak was investigated early and diseased animals were isolated and well managed to prevent further spread. Softening ointments and provision of soft and palatable feeds may help in more severe cases and help to keep intake up (Animal Disease Fact Sheet, 2007). Accordingly, broad-spectrum antibiotic ointment along with soft and palatable feeds was given to CE affected sheep to control secondary complications and to improve feed intake.

As the disease is commonly introduced into a sheep flock by replacement ewes or breeding rams and by contact with bedding material, trucks, and vehicles contaminated by the CE virus, strict adherence of CE prevention and control strategies is crucial (Leite-Browning, 2008). Vaccination for CE is a relatively simple procedure and should be done routinely in all but completely isolated flocks. However, it is known that once CE disease is entered and circulated in the sheep farm/flock, the virus is very hardy in the environment. It

can remain on the wool and hides for approximately one month after the lesions have healed and has been recovered from scabs after 12 years (CFSPH Technical Fact Sheets, 2006). Therefore, establishment of CE disease free flock with strict adherence to disease control and prevention strategies would help to effectively control CE in the study location.

CONCLUSION AND RECOMMENDATION

The PCR based assay proved that the outbreak that occurred at Adet Sheep Research Sub-Center was caused by CE virus with morbidity rate of 22%. As the present report is not exhaustive, detailed epidemiological study with phylogenetic analysis of CE virus should be conducted to know the genetic origin of the virus strain as well as for the future choice of CE immunoprophylaxis.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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