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Contagious ecthyma of sheep and goats: A comprehensive review on epidemiology, immunity, diagnostics and control measures

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ABSTRACT

Orf or contagious ecthyma is one of the contagious viral diseases responsible for economic losses to the countries rearing sheep and goats. This disease is characterized by its peculiar cutaneous lesions present mainly in the mouth region and less commonly on other sites, such as the abdomen and inguinal region. The unique immune-evasion properties of the orf virus results into short-term immunity both in natural infection as well as post-vaccination, as compared to other poxviruses associated with the presence of virulence genes namely VEGF, GIF (GM-CSF/IL2 inhibition factor), vIL10, VIR and apoptosis inhibitor etc. The disease mostly occurs in a mild form. However, the ability of the virus to cause repeated infection in the same animal, high morbidity in adults, mortality in young lambs and kids, its zoonotic nature, and its ability to cross-infect other species besides sheep and goats, make it an important disease globally. The disease is endemic to all the parts of India and causes marked economic losses. Diagnosis of orf mostly relies on conventional laboratory methods and few newer techniques are in use at present. Therefore, it is necessary to develop effective vaccines and update the diagnostic methods used for orf, to specifically identify the pathogen and subsequently design the control strategy. In this review, we focused on the current status of orf, its geographical distribution, economic impact, virulence genes associated with the virus, molecular epidemiology and related approaches for diagnostics, immuno-prophylactics, anti-viral therapeutics that have been developed, and future perspectives for the control of this contagious disease.

Key words: contagious ecthyma; small ruminants; zoonotic; epidemiology; diagnostics; vaccines; virulence genes; control

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Introduction

The orf virus is a prototypic member of the genus Parapoxvirus, of the subfamily Chordopoxvirinae and family Poxviridae (NANDI et al., 2011). It is known to cause a highly contagious and debilitating disease affecting the economy of many developing countries, including all the geographic regions of India (GUO et al., 2003; MONDAL et al., 2006; HOSAMANI et al., 2007; VENKATESAN et al., 2011; GELAYE et al., 2016). ORFV possesses a dynamic host range and infects a wide range of wild animals, such as the Japanese serow, musk ox, camels, reindeer, seals, and sea lions, along with its natural hosts, viz. sheep and goats (LANCE et al., 1983; TRYLAND et al., 2001; GUO et al., 2004; SHARMA et al., 2016). Humans contract the infection through direct contact with affected sheep and goats, or fomite contaminated with ORFV (LEAVELL et al., 1968) and it is considered an occupational zoonosis (ROBINSON et al., 1981). Generally, ORFV infection in humans is termed "farmyard pox". Orf is an English word which means 'rough' and the disease was termed so, because of the appearance of the skin after infection with the orf virus. The disease is highly contagious and affects small domesticated and wild ruminants (NANDI et al., 2011).

History

ORFV causing 'scabby mouth' in small ruminants has been known for a long time by farmers, but the etiological agent was identified and described (ROBINSON et al., 1982) in 1787. The first report of ORFV infection in sheep was submitted by Steeb in 1787, whereas the first description in goats was reported in 1879 (ARRANZ et al., 2000). ORFV's contagious nature and ability to infect humans were described later (HANSEN, 1879). Before 1956, the virus was known as a poxvirus-like disease-causing agent (DOWNIE and DUMBELL, 1956). A distinct group was described within the family Poxviridae by the International Committee on Nomenclature of Viruses (WILDY, 1971) in 1971. In 1976, parapoxviruses (pox-like viruses) were defined as one of the genera within the family Poxviridae by the ICTV (FENNER, 1976). Later, many similar viruses were identified and added to this genus.

Causative agent

The etiological agent, the orf virus (ORFV) is a prototypic member of the genus Parapoxvirus, subfamily Chordopoxvirinae, family Poxviridae. Other important members of the genus are pseudo-cowpox virus (PCPV), bovine papular stomatitis virus (BPSV) of cattle and the parapoxvirus of red deer in New Zealand (PVNZ) (KING et al., 2012). Apart from PVNZ, the other three species are identified with zoonotic potential. Some tentative species such as the chamois contagious virus, parapoxvirus of Japanese serow, musk ox, camels (Ausdyk virus), reindeer, seal and sea lions (KING et al., 2012) are yet to be characterized completely to be included under this genus. Recently, a novel poxvirus was isolated from a horse skin lesion, and its sequence analysis shows its relatedness with

members of the genus PPV, but more evidence is required to validate it as a new species in the genus (AIRAS et al., 2016).

Virion properties and Genomic organization

The ORFV virion with a size of ~260×160 nm shows a well-characterized ovoid structure that forms the basis of a separate group of PPVs within the family *Poxviridae* (ROBINSON et al., 1982). Negative staining electron microscopy reveals its unique criss-cross pattern, which is basically due to the superimposed images of spiral tubules surrounding the virion surface. This sealing of tubules around the virion is most probably due to a 10 kDa viral protein encoded by ORF104, a homolog of the vaccinia virus (VACV) A27L (SPEHNER et al., 2004). FLAG-tagged recombinant proteins showed that ORFV carries homologs of all structural genes of VACV in both infectious forms, except A36R, K2L, A56R and B5R (TAN et al., 2009) proteins. Regarding the physiochemical properties of ORFV, the virus is resistant to heat, ether and other lipid solvents, as reported for other poxviruses. Also, it survives for years in dry and extremely cold environmental conditions (ARRANZ et al., 2000) as the virus is trapped in an infected skin scab (HAIG and MERCER, 1998).

ORFV is a large enveloped dsDNA virus that replicates in the cytoplasm of the infected host cell. The genome size is ~135 kbp (MERCER et al., 1987), encoding approximately 132 proteins, with some variations among them. The central conserved region of ORFV (ORF009 to ORF111) is similar in order, orientation and spacing of genes as VACV, except the D9R and F15R genes (DELHON et al., 2004). They encode the proteins essential for viral replication, virion assembly, and morphogenesis. Terminal portions represent ~20% of the genome and comprise inverted terminal regions (ITRs) at both ends (up to 3kbp; around 31 genes) (DELHON et al., 2004; MERCER et al., 2006). Deletion of these terminal virulence genes is represented by an increase in ITR sequences in the genome, which is reported from 3kb to ~15-20kbp (FLEMING et al., 1995; COTTONE et al., 1998; McINNES et al., 2001). The remaining 70% of genes are common to other members of the subfamily Chordopoxvirinae. Characteristically, its high GC content (62-65%) presents more similarity with the Molluscum Contagiosum virus (MOCV) (DELHON et al., 2004). Phylogenetic studies also show this similarity on the basis of conserved non-structural genes such as DNA polymerase (GUBSER et al., 2004; XING et al., 2006).

Virulence genes or factors

Terminal regions of the genome contain dispensable genes for viral replication in cell culture, but support the survival of the virus in-vivo. These genes are known as virulence genes, and their products are virulence factors which help the virus to replicate and survive in a specific host immune environment (HALLER et al., 2014). Most of these genes are transcribed early in replication, except GM-CSF and the IL2 inhibition factor (GIF)

gene, and they modulate the host immune environment (JOHNSTON and McFADDEN, 2003) and are categorized under the pox-viral immune evasion (PIE) subfamily. Out of these genes, some are unique to PPVs and are responsible for determining the host range, pathogenesis, and virulence (FLEMIMG et al., 2015). Among these, the chemokine binding protein (CBP), GIF, viral interferon resistance (VIR), and dUTPase genes are of the VACV homolog proteins (C23L, A41L, E3L, and F2L respectively), and indicate their origin from the ancestral poxviral genes. However, the NF-kB inhibitors of ORFV are different from that of other poxviruses (FLEMIMG et al., 2015). The vascular endothelial growth factor (VEGF) gene is unique to parapoxviruses and not found in other members of the family Poxviridae, indicating that it is specifically acquired from their homologous host gene. GC content of a particular gene also indicates whether the gene belongs specifically to ORFV or was acquired from the host or other poxviruses (FLEMIMG et al., 2015). The low GC content of genes encoding VEGF and vIL-10 indicates that they are acquired from their host, while the high GC content of genes such as VIR and GIF (60-63%), indicates their specificity to ORFV. Because of the intricate relationship between the virus and its host during the course of evolution, some of the virulence genes are "captured" from the host during the course of evolution, such as dUTPase, vIL-10, VEGF, anaphase-promoting complex analog (PACR) and anti-apoptotic factors as indicated by their similarity to their cellular counterparts and absence from other poxviruses (FLEMIMG et al., 2015). These virulence genes have inter-isolate variations and help the virus to successfully infect highly specialized cells, *i.e.* keratinocytes, which are known as the first transducer of the signal for inflammation. These virulence genes, as a combined unit, are known to counteract the inflammatory immune response in infection. A more detailed study of these virulence genes could reveal the strategy of escape behavior of ORFV from the host defense mechanism, causing re-infection (HOSAMANI et al., 2009). Apart from virulence factors, recently ankyrin repeat proteins (ANK) encoded by ORFV have been identified as the up-regulating component for expression of cellular HIF, *i.e* hypoxia-inducible factors, by directly interacting with the factors inhibiting HIF and therefore promoting angiogenesis in the infected cells/organ (CHEN et al., 2017). The cyclophilin B (CypB) and peptidyl-prolyl cis-trans isomerase (PPIase) of the cell component are reported to be also involved in facilitating the replication of ORFV in the MDBK cell line (ZHAO et al., 2017).

Geographical distribution and economic impact

With its self-limiting nature and less economic impact when compared to other viral diseases of sheep and goats, orf has undefined epidemiological data and remains unreported in several countries (KUMAR et al., 2015). There have been several reports of Orf in both sheep and goats worldwide (NANDI et al., 2011) and also from different states of India (SOMVANSHI et al., 1987; VENKATESAN et al., 2011; MONDAL et al., 2006; HOSAMANI et al., 2007), Jammu and Kashmir (KUMAR et al., 2015),

Haryana (BATRA et al., 1999), Bihar (MUNDU and MOHAN, 1961), Uttar Pradesh (HOSAMANI et al., 2006), Assam (BORA et al., 2012; BORA et al., 2015), West Bengal (JANA and GHOSH, 2002; DEY and KUNDU, 2009), and Meghalaya (SHOME et al., 2005). The heterogeneity of circulating virus isolates or strains in India was described on the basis of A32L gene-based molecular epidemiology (YOGISHARADHYA et al., 2012). Although the orf is endemic in various countries, not much attention has been given to it as the disease is not included in the OIE (Office International des Épizooties) notifiable terrestrial disease list (GELAYE et al., 2016; ANONYM, 2017). However, orf is found under group 2 of OIE (Office International des Épizooties) listed diseases that are zoonotic and present in veterinary laboratories (ANONYM, 2017). The disease usually occurs in a mild form with a significant morbidity rate and productivity losses. The mortality rate is lower in adults whereas it may range from 10 to 90% in lambs and kids (MONDAL et al., 2006) due to their inability to suckle milk and secondary bacterial infections (VENKATESAN et al., 2011). Mixed infections of ORFV with PPRV (HOSAMANI et al., 2007, SARAVANAN et al., 2007), CaPV (VENKATESAN et al., 2014a; SELIM et al., 2016), Mycoplasma (CHU et al., 2011), and Streptococcus-Staphylococcus (CHI et al., 2017) have been reported. A report from England projected the production losses and treatment costs to be as much as $10 million and $4.62 per head based on 2.167 million affected sheep, indicating the significant trouble to the economy caused (BENNET and IJPELLAR, 2005). The global reported distribution of contagious ecthyma in different animal species is depicted in Fig. 1.

Fig. 1. Global distribution of contagious ecthyma in different animals, including sheep and goats (grey areas)

Epizootiology - susceptible hosts and transmission

ORFV mainly infects sheep and goats. It has also been reported in camels, alpacas, squirrels, and seals (HOSAMANI et al., 2009). There are reports describing ORFV infections in several other wild animal species, namely reindeer, musk ox, mule deer, white-tailed deer, pronghorn fawns and wapiti calves, by natural as well as experimental infection, showing its wide host range (LANCE et al., 1983; TRYLAND et al., 2001; GUO et al., 2004). Recently, a natural case of orf infection associated with sarcoptic mange in a free-range black buck was also confirmed by molecular diagnosis and B2L gene sequence analysis (SHARMA et al., 2016). ORFV also causes self-limiting localized pustular lesions in humans, mainly in the hands. ORFV may survive in a dry environment for months or even years (NANDI et al., 2011). Transmission mainly occurs through inanimate objects or fomites contaminated by the virus viz. grasslands, feeding troughs, equipment, workers etc. ORFV infection mainly aggravates by the end of the spring season ((ARRANZ et al., 2000). Persistently infected carrier sheep are probably responsible for the reappearance of orf within flocks and the transfer of orf between flocks (HOSAMANI et al., 2009).

Pathogenesis and clinical signs

The virus is epitheliotropic and replicates in regenerating epidermal keratinocytes. Despite the basic function of keratinocytes to provide protection from invading micro-organisms, ORFV successfully evades the defensive approach of the host (FLEMING et al., 2015). The infected epidermis is characterized by vacuolization and swelling of keratinocytes of the stratum basale. Infectious virions are detected 12 hrs after infection and the maximum titer has been reported to be between 24-72 hrs post-infection (LEAR, 1995). Lesions produced are of localized in nature and the scabs shed, to contaminate the environment, increasing the probability of affecting in-contact animals. Dendritic cells accumulated at the site of infections do not have access to T-cells in the lymph nodes, which is why the infection is restricted and localized (HAIG et al., 1997). Loss of epithelial integrity is the main predisposing factor in the initiation of the disease, along with stress factors, such as transportation, immune-suppression or primary infections (NANDI et al., 2011). Lesions are mostly confined to the epithelium of the oral mucosa, the skin of the lips and around the nostrils, but it can also be found on the teats of nursing animals, and rarely on the tongue and gums of affected animals (HOSAMANI et al., 2007; 2009). Clinically, the disease is characterized by proliferative and usually self-limiting lesions that progress through stages of erythema, papule, vesicle, pustule, and scabs, as observed in other pox viruses (KARAVAGLIO and KHACHEMOUNE, 2017). These lesions are painful, highly vascular in nature, and the scabs are friable and bleed easily (BARRAVIERA, 2005). The disease usually lasts for 3-4 weeks and lesions resolve in 1-2 months, with the shedding of scabs without leaving a scar (NANDI et al., 2011).

Host immunity and its evasion by virus

Immunity developed against the ORFV is short-lived, and so far there is no clear or complete understanding of the mechanism of protective immunity. There are many discussions over the type of immunity that plays a major role in ORFV infection (McKEEVER et al., 1987; YIRREL et al., 1989). Many have suggested that humoral immunity does not play a major role in protection, as observed earlier (AYNAUD, 1923; BUDDLE and PULFORD, 1984), and the antibody titre merely indicates a previous infection. However, some studies have shown that a specific isotype (IgG2) plays an important role in infection (HAIG et al., 1996).

Studies have been conducted to unravel the status of immune cells in the skin of primary infected and re-infected sheep (BOS and KAPSENBERG, 1993). It was demonstrated that neutrophils, T-cells, B-cells, and dendritic cells are the prime cells in the infected keratinocytes (JENKINSON et al., 1990). Among all, CD4+ T cells were found to be the predominant cells in lesions from the primary and re-infected animals. In spite of the host's defense system, the virus is able to re-infect the host successfully. The lesions in re-infection are reported to be smaller and resolve sooner. It is hypothesized that IL-2 and IFN-ɣ cytokines play a protective role during re-infection (HAIG et al., 1997).

Zoonotic potential

Orf is an emerging viral zoonosis, with an increasing number of outbreaks reported worldwide, and is a contagious disease that tends to spread very quickly among livestock and animal handlers (DEMIRASLAN et al., 2017). Most cases of orf are reported in farmers, veterinarians, and zoo personnel, while shearing, slaughtering, drenching affected animals, as an occupational disease (ARRANZ et al., 2000; ESSBAUER et al., 2010; HASHEMINASAB et al., 2016). Although its ability to infect humans was mentioned by Hansen in 1879, the first case of the disease in humans was described in 1934 (GROVES et al., 1961). Lesions in humans usually develop on the fingers, hands or forearms, and are benign in nature (PAIBA et al., 1999; CUBELLS et al., 2016). The systemic forms of infection, such as erytema multiformis and generalized lymphadenopathy are very rarely seen (BIAZAR, 2016). A rare report of a patient with an orf lesion on the nose was documented recently in a Muslim country after a ritual feast (ATA et al., 2017). Also, a case of orf infection in a burns patient in Iran was reported, and its phylogenetic relatedness with an Indian isolate was unexpectedly confirmed (HSU et al., 2016). Also, the risk of zoonotic orf can be estimated from a clinical case report which discusses multiple orf lesions in the fingers of a 13 month old child (HADDOCK et al., 2017). Cases of human to human transmission are very rare and have not been reported so far (DEMIRASLAN et al., 2017). Infection is generally self-limiting, but may create more serious effects in immuno-compromised persons. MNB029/98 is a well-defined human

biopsy-derived strain, collected from the owner of a contagious pustular dermatitis infected flock, which can be used for comparative analysis in the case of human infections (FRIEDERICHS et al., 2014).

Diagnostics tools and methods

Acute and proliferative pustular cutaneous lesions (VENKATESAN et al., 2011) that are mainly present on the skin of lips, oral commissures, oral mucosa and nostrils (Fig.1 A&B) are typical clinical findings. Histopathology reveals specific changes such as vascular degeneration and swelling of keratinocytes (POZZO et al., 2011). The similar lesion producing diseases such as CaPV, FMD, Bovine herpesvirus type-2, and bluetongue virus, mean differential diagnosis of this disease is necessary at the molecular level (NANDI et al., 2011). Therefore, specific and sensitive diagnostic tools including recombinant protein based ELISA can meet the need of public health workers to eliminate the chances of misdiagnosis and provide a confirmatory diagnosis to formulate correct therapeutic management (WATSON et al., 2002).

Virus isolation. The primary lamb kidney (PLK) and primary lamb testis (PLT) are the most commonly used cells for virus isolation (PLOWRIGHT et al., 1959). Other cell lines include OA3.Ts, Vero, ovine organotypic skin culture, ovine fetal turbinate (OFTu) cells, BHK-21, and Madin-Darby ovine kidney cells (SCAGLIARINI et al., 2005). Among these cell lines, the maximum titre was recorded in the primary cell line derived from sheep, suggesting the suitability of ovine primary cell lines for multiplication of the virus (IVANOV et al., 2016). The permanent cell lines also favor the multiplication of the virus, but titre yield is comparatively lower. Within 2-3 days, the virus titre reaches its maximum, and typical cytopathic effects such as rounding, pyknosis, ballooning and detachment of cells, are observed at the same time. The chorio-allantoic membrane of embryonated chicken eggs may also be used for virus isolation (ZEEDAN et al., 2015). Negative electron microscopy is the method of choice to differentiate ORFV from other poxviruses (HAZELTON and GELDERBLOM, 2003).

Serological techniques. Counter immunoelectrophoresis (CIE) is also a commonly practiced laboratory technique for orf diagnosis, but it cannot give a confirmatory diagnosis since CaPV and ORFV share some common soluble antigens (SUBBA et al., 1984). Therefore, CIE results need to be confirmed with molecular techniques such as PCR, real-time PCR and gene sequencing. The serological tests used for detection of anti-ORFV antibodies include the virus neutralization test (SNT), agar gel immunodiffusion (AGID) (TRUEBLOOD et al., 1963), complement fixation test (CFT) (GLOVER, 1933), and the Fluorescent agglutination test or agglutination (NANDI et al., 2011). A titre of 8 and 20 or above can be considered positive for orf infection by SNT and CFT, respectively (ZARNKE et al., 1983). Virus neutralization assay is considered the gold standard for poxvirus sero-diagnosis, but its lower sensitivity is a drawback.

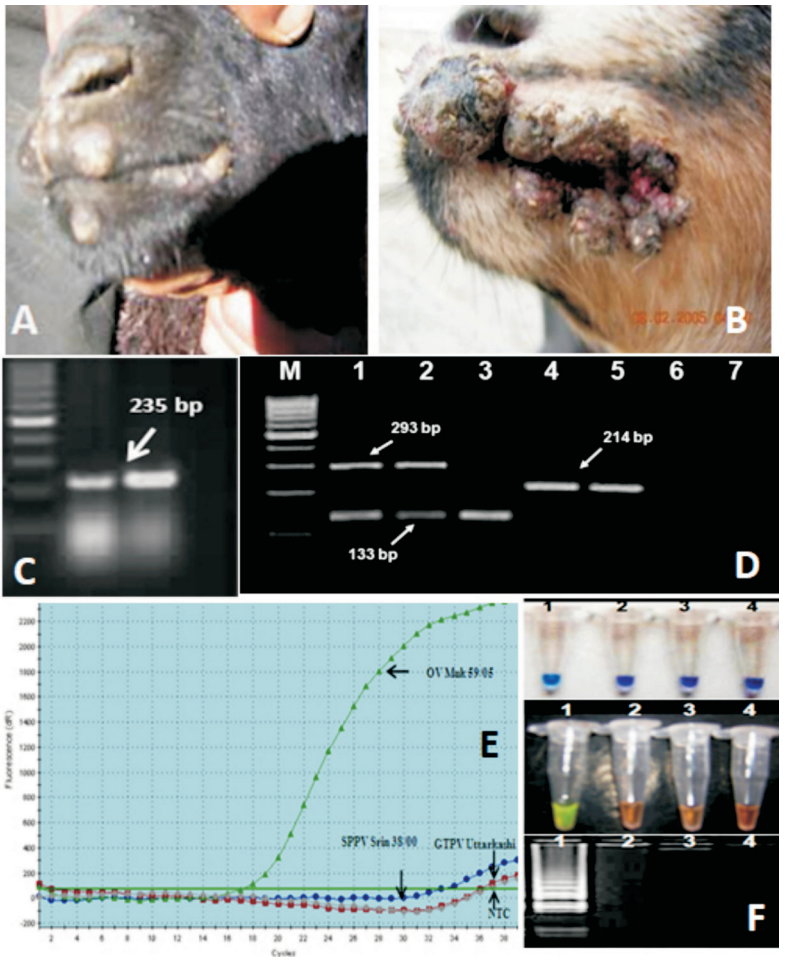

Fig. 2. Panel A&B: Characteristic localized proliferative and button type skin lesions around the mouth in sheep and goats; Panel C: Semi-nested diagnostic PCR, showing specific amplification of B2L amplicons of 235 bp size from ORFV suspected samples (Lane 1&2) and negative control (Lane 3); Panel D:Multiplex PCR showing detection and differentiation of ORFV (Lane 4-5: 214bp) from SPPV (Lane 1-2: 293 bp& 133 bp amplicons) and GTPV (Lane 3: only 133 bp); Panel E: DNA Polymerase gene based TaqMan real-time PCR for specific and sensitive detection and quantification of ORFV in clinical samples; Panel F: LAMP assay showing rapid and specific detection of ORFV (Lane 1), as positive from sheeppox virus (Lane 2), goat pox virus (Lane 3) and NTC (Lane 4) as negative controls by Ladder pattern in agarose gel and addition of HNB and SYBR green I dyes

The results of AGID and FAT are also not as completely reliable, like the CIE test, as ORFV share some common antigens with CaPV (KITCHING et al., 1986). Western Blot analysis (WB) has been used for confirmation of ORFV infection based on two immunodominant envelope proteins, viz. 39 kDa and 22 kDa (CZERNY et al., 1997; GUO et al., 2003; LI et al., 2012b). WB analysis based on the antibody response to the P32 antigen of CaPV has been established and offers serological differentiation from ORFV (CHAND et al., 1994). ELISA using the whole virus and subunit antigens of the ORFV virion has been reported to detect ORFV antibodies earlier (CHIN and PETERSEN, 1995). Anti-ORF086 (WANG et al., 2015) and ORFV059 MAb (LI et al., 2012a) have been successfully developed and proven to be strong candidates for developing diagnostic tools and evaluating ORFV pathogenesis more accurately. Another on-site diagnostic technique, lateral flow immunochromatographic assay (LFIA), has been developed for orf using two monoclonal antibodies against ORF011 protein (ZHAO et al., 2016).

Molecular technique. Molecular detection and characterization of members of PPV, including ORFV, was initially based on restriction fragment length polymorphism (RFLP) and nucleic acid hybridization, which revealed genetic heterogeneity among isolates of the same viral species (WITTEK et al., 1980; ROBINSON et al., 1982; GASSMANN et al., 1985; RAFII and BURGER, 1985). As RFLP and hybridization procedures are cumbersome and less sensitive, several PCR protocols have been developed for rapid detection of PPV members, including ORFV, to avoid use of cross-reactive serological tests, highly tedious cell culture systems, and less sensitive electron microscopy (MAZUR et al., 2000; INOSHIMA et al., 2000; 2001; 2002; GUO et al., 2003; 2004; TORFASON and GUONADOTTIR, 2002; TRYLAND et al., 2005). ORFV-B2L gene encoding a 45kDa envelope protein is a highly immunogenic, conserved gene that has been targeted for identification of ORFV in diagnostic PCRs (HOSAMANI et al., 2006; VENKATESAN et al., 2011; BORA et al., 2012; 2015) Semi-nested PCR for specific and sensitive diagnosis of ORFV in clinical samples is shown (Fig. 2C). Also, the VIR gene and ORF045 were reported to be effective for the identification of ORFV (GUO et al., 2004; KOTTARIDI et al., 2006). Highly sensitive multiplex PCR (mPCR) targeting the I3L (DNA binding phosphoprotein) gene of CaPVs and the E9L (DNA polymerase) gene of ORFV has been developed for simultaneous detection and differentiation (Fig. 2D) (VENKATESAN et al., 2014a). Similarly, duplex PCR, targeting fragments of the A29L gene of CaPV and the P55 gene of ORFV, has been developed for easy differentiation (ZHENG et al., 2007). Initially, pan-pox PCR assays were also developed to separate the two groups of poxviruses, as a high GC content and a low GC content virus (LI et al., 2010).

Real-time PCR based on ORF056 (Vaccinia virus J6R homolog) has been validated to differentiate different viruses from the same genus (INOSHIMA et al., 2000). A highly sensitive SYBR Green I based real-time PCR was reported for identification of ORFV

genomic DNA in 1.5 hrs (WANG et al., 2017). TaqMan based real-time PCR has also been developed for specific and sensitive identification of ORFV, targeting the B2L gene (YANG et al., 2006), the ORF024 gene (DU et al., 2013) and the DNA polymerase gene (BORA et al., 2011). DNA polymerase gene-based TaqMan probe real-time PCR was found to be specific and rapid to detect and quantify ORFV in suspected clinical samples (Fig. 2E) (BORA et al., 2011). A genus-specific (Parapoxvirus) real-time PCR using the TaqMan probe was recently developed targeting the DNA polymerase gene (DAS et al., 2016). As an alternative to PCR, LAMP assay has also been proved to be effective in diagnosing various diseases in field conditions (NOTOMI et al., 2000). LAMP assays targeting the B2L gene (TSAI et al., 2009; VENKATESAN et al., 2016), the F1L gene (WANG et al., 2016) and the DNA polymerase gene (LI et al., 2013; VENKATESAN et al., 2015) have been successfully optimized to detect ORFV (Fig. 2F). The sensitivity and specificity of the LAMP assay, its easy handling, and the less time needed, suggest LAMP as a comparatively more reliable technique than PCR (VENKATESAN et al., 2015). A fluorescent probe-based assay which gives results within 20 min at 37 °C, using the specific detection probes is highly specific and sensitive for ORFV, and has been found to be comparable to other 'pen-side tests', like LAMP (YANG et al., 2006). Recently, LAMP assay using Bst or Csa polymerase with HNB as an indicator was developed successfully to diagnose orf in Japanese serows (INOSHIMA et al., 2016). A list of various molecular diagnostics for detection of PPVs is shown in the Table 1.

Table 1. List of nucleic acid based molecular diagnostic tools and methods used in rapid detection and differentiation of PPVs

SL. No.	Diagnostic assay or method	Gene (s) targeted	Key point(s) in diagnosis	Reference
1.	PCR	B2L (ORF 011)	Pan-parapoxvirus PCR; Same primers along with two inner primers were used for semi-nested PCR also	Inoshima et al., 2000
		ORF101 (RPO30)	Used for both animal and human diagnosis of orf	Torafson and Guonadottir, 2002
		VIR (E3L)	Used in detection and characterization of ORFV isolates from different zoo species	Guo et al., 2004
		ORF045 (VLTF-1)	Found to be more sensitive than B2L and VIR PCRs	Kottaridi et al., 2006
		A32L	For genotyping of ORFV on the basis of c-terminal heterogeneity of A32L gene sequence	Chan et al., 2009
2.	Semi-nested PCR	F1L	Differentiation of ORFV strains from SPPV and GTPV	Yu, 2014

Table 1. List of nucleic acid based molecular diagnostic tools and methods used in rapid detection and differentiation of PPVs (continued)

SL. No.	Diagnostic assay or method	Gene (s) targeted	Key point(s) in diagnosis	Reference
3.	Multiplex PCR	I3L for CaPV E9L for ORFV	Detection and differentiation of SPPV, GTPV and ORFV in sheep and goat scab samples	Venkatesan et al., 2014a
	Duplex PCR	A29L of CaPV P55 of ORFV	Specific and sensitive detection with a limit of 1 pfu	Zheng et al., 2007
4.	Real time PCRs			
	SYBR Green I based real time PCR	B2L	Can detect minimum of 20 copies of genomic DNA in 1.5 hrs	Wang et al., 2017
	TaqMan based real-time PCR	DNA Polymerase	Rapid detection of ORFV in clinical samples	Venkatesan et al., 2012
		B2L	Better and easy quantification of virus for testing antiviral agents	Gallina et al., 2006
		ORF024	Sensitivity 5fg or 15 copies of genomic DNA	Du et al., 2013
		DNA polymerase	ORFV specific PPVs specific and highly sensitive	Bora et al., 2011 Das et al., 2016
		RNA polymerase	Generic and specific detection of PPVs	Zhao et al., 2013
	Duplex real-time PCR	DNA polymerase of CaPV and ORFV	Taq Man duplex Real-time PCR for simultaneous detection and differentiation of CaPV and ORFV in sheep and goats	Venkatesan et al., 2014b
5.	LAMP assay	DNA Polymerase B2L F1L	Visual detection of ORFV suspected samples Simple visual diagnosis of ORFVs Rapid detection of CE in clinical samples	Li et al., 2013 Tsai et al., 2009 Wang et al., 2016
6.	Fluorescent probe based assay	DNA polymerase	within 20 min at 37 °C using the specific detection probes	Yang et al., 2006

Molecular epidemiology of ORFV

Genetic characterization of ORFV strains or isolates from a particular geographical location targeting the structural or non-structural, or immunomodulatory genes of the virus can provide the phylogenetic relationship and genetic variations among circulating strains. It will also give sufficient information to initiate preventive and control measures for spreading infections in susceptible hosts. It may also provide strategies for development of molecular diagnostics, subunit vaccines and anti-viral therapeutic agents to control the infection. Among the structural protein genes used in the molecular epidemiology of ORFV and other PPV infections, the B2L (ORF011, an immunogenic envelope protein) gene is used most frequently in genetic analysis and development of molecular diagnostic tools (INOSHIMA et al., 2000; HOSAMANI et al., 2006; VENKATESAN et al., 2011; BORA et al., 2011; CHI et al., 2017). Other genes, such as ORF 059 (F1L), ORF 020 (E3L/VIR), ORF 117 (GUO et al., 2004; HOSAMANI et al., 2007) and ORF032 and ORF 080 (CHI et al., 2017) are used in the phylogenetic study of PPVs, including ORFV isolates. PPVs, including ORFV associated virulence factors, are VIR, NF-kB inhibitors, CBP, GIF, dUTPase, PACR/ring H2 protein, VEGF, viral Interleukin-10 (vIL-10), inhibitor of apoptosis etc. The conserved functionality of these genes, due to the presence of some functionally conserved domains in spite of the marked sequence variations, is responsible for the activity of encoded virulence factors, establishing them as promising candidates for further studies (KARKI, 2017). Detailed characterization of these virulence genes in the future may unravel the mechanism of the immune evasion strategy of ORFV, and may provide the co-evolutionary process of the virus with the host in the process of developing resistance to immunity and persistence in the same infected hosts.

Vaccines

Vaccination is the ultimate solution to control and eradicate any infectious and contagious viral disease from an endemic country. Conventional vaccines have been found to be ineffective for orf as demonstrated by an *in-vivo* assay, where administration of inactivated ORFV in a host produced lesions on challenge and did not even prevent re-infection of the host (McGUIRE et al., 2012). So far no vaccine is commercially available which gives 100% protection against ORFV infection. Therefore, its control remains a challenge and target hosts are under threat. Dermal scarification, with scabs infected with a high titre of ORFV, has been used to confer some immunity, but for a short period of time (HOSAMANI et al., 2009). The first vaccine for contagious ecthyma was developed in Texas for sheep (BOUGHTON and HARDY, 1935). There was one study which suggests that distinct clusters of sheep and goat ORFV strains exist, and more heterogeneity within goat strains was reported in phylogenetic analysis (MUSSER et al., 2008). This may account for the inefficiency of vaccines derived from sheep ORFV

strains to prevent the infection in goats or vice-versa. The caprine ORFV strain (47CE) has been demonstrated to potentially provide immunity to goats against wild type ORFV infection (MUSSER et al., 2008). A vaccine using primary lamb testis cells, developed in the Commonwealth Serum Laboratories, Australia, has validated protection for 6 months (NETTLETON et al., 1996). Also, OKA and NARA strains have been used to develop a cell culture-adapted ORFV vaccine, replacing the conventional sheep-grown virus vaccine (PYE, 1990). In India, live attenuated orf vaccine (Mukteswar 59/05 strain) passaged in primary lamb testicle (PLT) cells has been developed at IVRI, Mukteswar, and has been found to be safe, efficacious and potent in sheep and goats (BHANUPRAKASH et al., 2012). A novel approach by cloning the gene fragment of ORFV into the VACV vector, showed protection against infection (MERCER et al., 1997). Also, a safe DNA vaccine, expressing ORFV 011 and 059 chimeric proteins, has been developed, showing great improvement in immunogenicity and the potency of vaccination (ZHAO et al., 2011). Recombinant goatpox virus expressing the F1L gene of ORFV was constructed and proved to be a stable vaccine to provide protection against both GTPV and ORFV (ZHANG et al., 2014). B2L, a highly conserved and immuno-dominant envelope protein, was found to elicit dendritic cells and other immune cells at the site of infection, and is presented as an appropriate candidate for vaccine development (ZHAO et al., 2011). *In-vivo* gene delivery, directly assessing its functions, is a novel method for detecting vaccines and therapeutic applicants rather than isolation, cloning, characterization and then identifying a protective antigen. This study revealed the activity and protection of the B2L gene constructed in influenza gene-vaccine in mice (McGUIRE et al., 2012).

Recombinant ORFV

Due to the large genome size, ORFV also presents suitable genomic sites for developing novel recombinants for developing diagnostics and viral vectored vaccines (ROBINSON et al., 1982). The immuno-modulatory activity of ORFV makes it an efficient viral vector and, therefore, studies were conducted to replace the ORF024 and ORF121 genes with rabies virus G protein, and it had recognized and appreciable immunogenicity (MARTINS et al., 2017). ORFV-D1701 is a sheep-derived strain obtained after 135 passages in cell culture to reduce its virulence and pathogenicity (MAYR et al., 1981). Attenuated ORFV-D1701 has also been approved in many countries to be used as a live vaccine for orf (MAYR et al., 1981). ORFV-D1701 shows a genome size of approximately 158 kbp, which is comparatively larger than earlier published genomes of ORFV isolates (COTTONE et al., 1998). Genomic studies on the D1701 strain showed an increased ITR region up to 18 kbp due to the duplications and genomic rearrangements that occurred with the increase in cell culture passages (RZIHA et al., 1999). Also, the E2L gene was reported to be absent from the D1701 genome, suggesting its non-essential role in viral replication and making it a potential target (RZIHA et al., 1999). Regarding

the immune-potential of this attenuated virus, it stimulates the activation of IFN-α, IL-1β, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF), Th-1 and IFN-γ, but lacks the immune evasion strategies possessed by active ORFV (WEBER et al., 2013). Therefore, it has the capability to induce short-term non-specific immunity, termed as a paramunity inducer (BERGQVIST, 2017). Baypamun® or Zylexis® are commercial products of the attenuated ORFV D1701 strain licensed in the UK and used as an immunomodulator to potentiate immunity in horses against infectious respiratory diseases (ZIEBELL et al., 1997). Further, the inactivated ORFV-based immunomodulator has also been used to treat bovine respiratory disease (ZIEBELL et al., 1997), post-weaning diarrhea syndrome (KYRIAKIS et al., 1998), chronic stomatitis in cats (MAYR et al., 1991) mammary tumors in dogs (BERG and RUSSE, 1994) and the genital herpes virus (FRIEBE et al., 2017).

Anti-ORFV agents/therapeutics

The ability to manipulate the host immune system, as well as the non-availability of efficient vaccines for control of orf infection, makes the antiviral therapeutics a better alternative to counteract this contagious disease. Many antiviral agents, such as nucleoside/nucleotide analogs and their phosphonate/alkyloxy ester derivatives, have been evaluated and used for poxviruses (SNOECK et al., 2002; SMEE and SIDWELL, 2003; DE CLERCQ and NEYTS, 2004; LEBEAU et al., 2006). The orf virus has also been found to be sensitive to acyclic nucleoside phosphonates in the cell monolayer, as marked by histopathology and DNA quantification methods (DAL POZZO et al., 2005). Among all the analyzed antivirals agents for poxviruses, the activity of cidofovir was found to be superior for PPVs (NETTLETON et al., 2000). The efficacy of cidofovir against PPVs was found to be much higher than vaccinia virus, which therefore makes it an effective therapy to treat prolonged and complicated ORFV lesions in humans (NETTLETON et al., 2000). Topically cidofovir (1%) has been proved to treat cases of orf lesions in children and immunocompromised patients (GEERINCK et al., 2001; McCABE, 2003) as well as in veterinary medicine trials (SCAGLIARINI et al., 2007). Different formulations of cidofovir and cidofovir-sucralfate gel suspension have proved to be excellent topical agents in resolving orf lesions in lambs (SCAGLIARINI et al., 2007; SONVICO et al., 2009). Moreover, the alkyloxy ester derivatives of cidofovir were found to possess more oral bioavailability and cause less damage to kidneys than the parent compound used (DAL POZZO et al., 2007). A comparative study suggested that the high GC content may be a linking trait for the sensitivity of ORFV to cidofovir, as the same was also reported for molluscum contagiosum and herpes viruses (NETTLETON et al., 2000; TEMPESTA et al., 2008).

Conclusion and future perspectives

The mild form of the disease and its associated outcomes are the reason that to date there has been a lack of proper surveillance, epidemiological data and effective control strategies for the disease, despite the economic losses it causes in most developing countries rearing sheep and goats. Therefore, proper surveillance implementation and infection control measures are very important for prevention of orf infection. Even then, its endemicity in many countries, wide host range, zoonotic potential, short-term immunity, and, most importantly, its unique immune evasion property as compared to other poxviruses, makes it an important pathogen to be studied expansively. ORFV is stable in all types of agro-climatic conditions and therefore spreads very easily, leading to frequent outbreaks. In field conditions, the lesions are often confused with other important diseases such as capripox, bluetongue, foot and mouth disease, mange, dermatophilosis etc. So far there have been no recombinant antigen based diagnostics for ORFV, which is a major lacunae in its diagnosis in differentiating the disease. Genetic characterization of virulence genes will help in determining the molecular epidemiology and phylogenetic relationship among members of the genus, and may unravel the mechanism of the immune evasion strategy of ORFV in future. For example, the VEGF gene has been found to be responsible for the peculiar finger-like projections of the lesion (rete-ridges), and scab formation, and deletion of this gene has resulted in a marked decrease in the severity of the disease, indicating its importance in virulence. Studies will also help to establish the epidemiological distribution of ORFV in India by identifying genetic relatedness or variations among circulating virus isolates/strains in diverse geographical areas. ORFV provides short-term immunity as compared to other members of Poxviridae. Dispensable sites within the gene or whole genes, viz. VEGF, may be targeted to develop vectored vaccines and gene-deleted/marker vaccines. Further, the development of sensitive and accurate diagnostics is a prerequisite to differentially diagnose diseases and subsequently plan control strategies for important diseases. Also, there is a need for low cost, safe and efficacious alternatives to conventional vaccines to control the disease in the future.

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SAŽETAK

Zarazni ektim ovaca i koza jest virusna zarazna bolest koja uzrokuje gospodarske gubitke u zemljama koje se bave uzgojem ovaca i koza. Bolest je obilježena specifičnim kožnim lezijama, najčešće u području usta te, rjeđe, na drugim dijelovima tijela poput trbuha ili preponskog područja. Jedinstvena sposobnost orfvirusa za izbjegavanje imunosnog odgovora rezultira kratkotrajnom imunošću i u prirodnim infekcijama i poslije vakcinacije, a u usporedbi s drugim poksvirusima taj odgovor povezan je s prisutnošću gena virulencije VEGF, GIF (GM-CSF/IL2 faktor inhibicije), vIL10, VIR i inhibitorom apoptoze. Bolest se većinom pojavljuje u blagom obliku. Bez obzira na to, sposobnost virusa da uzrokuje ponovljene infekcije u iste životinje, visok pobol u odraslih jedinki, smrtnost u mlade janjadi i jaradi, zoonotska narav bolesti te sposobnost da uzrokuje križne infekcije u drugih vrsta osim ovaca i koza, čini ovu bolest važnom na globalnoj razini. Bolest je endemijska u svim dijelovima Indije i uzrokuje znatne gospodarske gubitke. Uglavnom se dijagnosticira konvencionalnim laboratorijskim metodama, a trenutačno se primjenjuje i nekoliko novih metoda. Potrebno je stoga razviti učinkovita cjepiva te poboljšati dijagnostičke metode koje će biti specifične za dokaz patogena i na kojima će se temeljiti mjere kontrole. U ovom radu pozornost je usredotočena na aktualno stanje u pogledu orfvirusa, njegovu zemljopisnu raširenost, gospodarski utjecaj, gene virulencije povezane s virusom, molekularnu epidemiologiju i pristupe u dijagnostici, imunoprofilaksu, dosad razvijene antivirusne lijekove te buduće perpsektive u kontroli ove zarazne bolesti.

Ključne riječi: zarazni ektim; mali preživači; zoonoza; epidemiologija; dijagnostika; cjepiva; geni virulencije; kontrola

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