Emergence and Reemergence of Vaccinia-Like Viruses: Global Scenario and Perspectives

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Abstract Among the members of the genus *Orthopoxvirus* (OPXV), vaccinia virus (VACV), the type species of the genus is a double-stranded DNA virus, belongs to the subfamily *Chordopoxvirinae* of the family *Poxviridae*. The causative agents of smallpox, VACV and Variola virus are mutually immunogenic and the type species of *Orthopoxvirus*, cause only mild complications in humans. Therefore, the VACV was used as a smallpox vaccine world over under mass immunization program promoted by World Health Organization, which lead to the variola eradication globally in 1979. Since then, no vaccination of human population has been carried out; however, vaccination has been continued for at-risk laboratory workers, military personnel and others working with recombinant VACV or other non-variola orthopoxviruses (OPXVs). There has now been a surge in the development of safer smallpox vaccines and understanding of the biology of VACV necessitating re-use of this vaccine in most vulnerable population, because of rise in bioterrorist threats globally. Also, globally there has been the emergence and re-emergence of vaccinia-like

viruses (VLVs) in Brazil, buffalopox viruses in Egypt, Indonesia, India and its neighbouring countries like Nepal, Pakistan. Bioterrorism as well as emergence and re-emergence of the VLVs constitute a concern as 50 % of the population globally (40 % in USA) <30 years are unvaccinated and most vulnerable for smallpox reemergence. Thus, the search for new generation safer smallpox vaccine entails review of biology of VLVs in the smallpox-free world. In this review, we present occurrence of VLVs in the world with exhaustive discussion particularly on the emergence and re-emergence of these viruses in India and Brazil where VLVs are sufficiently studied.

Keywords Vaccinia virus · Vaccinia-like viruses · Buffalopox · Emergence and reemergence · Zoonosis

Introduction

Since smallpox eradication in late 1970s, there have been rare reports on outbreaks of human infections by poxvirus. Different poxviruses such as Cowpoxvirus (CPXV) and vaccinia virus (VACV) (genus: *Orthopoxvirus*); Orf virus, Bovine Papular Stomatitis and Pseudocowpox (genus *Parapoxvirus*); and Bovine Herpes 2 (*Herpesvirus*) can cause very similar lesions on udders of cows and hands and arms of dairymaid. The etiological diagnosis of these diseases, commonly known as “disease of dairymaid”, “bovine variola”, “pseudovariola” or still “paravaccinia” is difficult to be established authentically [25]. Outbreaks initially attributed to CPXV were identified as caused by VACV, which is type species of the genus *Orthopoxvirus* (OPXV), a double-stranded DNA virus, belongs to the subfamily *Chordopoxvirinae* of the family *Poxviridae* [42]. VACV had been in use as a smallpox vaccine world over since long

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period under mass immunization program promoted by World Health Organization (WHO), due to its similar immunogenic relationship to Variola virus (VARV) which led to the smallpox eradication from the world in 1979 [12]. But, it is also recommended to use for laboratory workers and military personnel under risk of contracting smallpox and other persons handling unattenuated and recombinant VACV or other non-variola OPXVs namely monkeypox. There is a demand in development of safer smallpox vaccine and abreast the knowledge on molecular biology of VACV in recent past due to global threat of bio-terrorist activities, re-use of smallpox vaccine in certain susceptible population as mentioned earlier and emergence of vaccinia-like viruses (VLVs) namely buffalopox in Asia and other VLVs in Brazil. Among these, bioterrorism and emerging form of VACV variants constitute concern for more than 50 % of population worldwide. Cessation of vaccination against smallpox since 1980s, emergence of some genetically related OPXVs has also been reported throughout the world i.e. monkeypox, buffalopox and bovine vaccinia infections [43]. In addition to a close variant of VACV called as buffalopox virus (BPXV) circulating among buffaloes, cows and human in India, there were many reports of emergence of human and animal pox infections in other parts of the world namely VACV like agents called Aracatuba virus (ARAV) and Cantagalo virus (CTGV) in cattle, humans [9, 39] and wild rodents [1] have been reported in Brazil. There has been increase in the incidence of vaccine like virus infections in some parts of the world affecting not only animals but also humans. Therefore the review focuses on VLVs existing worldwide, their origin, molecular epidemiology and global impact on veterinary and public health.

Human VLVs

Reports on human poxvirus infections have been rare since smallpox eradication. The main OPXVs that infect human include VARV and Monkeypox virus (MPXV) as seen in Congo and USA, CPXV in Europe and VACV in India which was the vaccine strain used for smallpox eradication [25]. The CPXV and VACV cause similar local lesions in humans upon contact with infected cow. Pox disease in human reported from countries like Brazil [25] and India [33] has usually been observed in milkmaids/men, laboratory personnel handling the virus, or persons involved in the husbandry of cows and buffaloes.

Existence of VLVs in the World

Buffalopox exists in India since 1934 and disease outbreaks have still being occurring in buffaloes, cows and humans

[36]. Buffalopox is caused by BPXV a variant of VACV classified under G: *Orthopoxvirus* of the S.F. *Chordopoxvirinae* and F: *Poxviridae* [24]. Extensive isolation and characterization studies using conventional techniques were unable to differentiate between BPXV and VACV [30, 31]. Of late, we have been able to generate sequence information on a few genes of the BPXV isolates, employing field isolates from buffaloes and cows from 1996 till 2009 (Table 1). The reference strain BP4 was isolated originally in Hisar, India. Similarly, pox-like outbreaks in cows and humans have also been occurring in Brazil for quite long time. Recently, the viral agents causing these outbreaks were isolated and genetically characterized establishing them as VLVs [25, 40]. The causative agents for these outbreaks have been identified as SPAn-232, BeAn-58058 (BAV), ARAV, CTGV, *Belo Horizonte* virus (VBH) in Brazil, while, buffalopox in India, Nepal, Pakistan, Egypt and Indonesia (Table 1; Fig. 1).

Pox-like cases in buffaloes and humans have been recorded in India regularly [33]. However, there are only a few studies in these viruses not only in Brazil and India, but also throughout the world. The points to be considered are (i) epidemiologic surveillance with a seroepidemiology that would help to determine the transmission chain, (ii) molecular epidemiological studies to establish the real identity of the pox-like viruses circulating in a particular geographic area and (iii) to identify the natural host of these viruses as this zoonosis is detrimental to the local economy and public. Now it is established that the VLVs reported from animals and humans in Brazil are variants which arose from VACV (smallpox vaccine). A few VLVs, which have been characterized lately, are being discussed in the succeeding text.

BPXV, a VLV in India

Buffalopox, is being reported continuously from India [33], which is primarily a disease of buffaloes caused by BPXV [44], a subspecies of VAVC—that causes localized lesions in milking buffalo, dairy cattle and oropharyngeal lesions (drinking unpasteurized milk) in humans [9, 11]. Buffalopox is an important zoonosis. Clinically, BPXV lesions are akin to cowpox vesicles, though less painful, but lesions of mild cases resemble VACV vesicles. BPXV outbreaks were recorded during the smallpox vaccination in India, Egypt, and Indonesia and also later from India [20, 33], Pakistan (Qureshi, Personal communication) and Nepal [44]. Recently, analysis of samples collected from cows suffering with pox-like disease during 2002–2006 throughout India revealed that cows suffered from BPXV mostly than CPXV based on sequence and phylogenetic analyses of HA gene

Table 1 Current status of emergence of Vaccinia-Like Virus (VLVs) in India and Brazil

Year of outbreak	State	Remarks	Reference
VLV Buffalopox virus in India			
1934	Lahore in undivided India	Pox disease in buffaloes	–
2003	Aurangabad (MH)	Both the buffaloes and human (Milkers) were affected	[33]
2005–2006	Tamil Nadu	Buffaloes and human (Milkers) were affected. BPXV characterized	Unpublished data
2008–2009	Aurangabad (MH)	Zoonotic buffalopox outbreak occurred. Virus isolates of both buffalo and human were isolated and characterised	[4]
2009–2010	Pune, Maharashtra	Severe buffalopox zoonosis reported and characterization of virus isolates of both origin have been carried out	[44]
VLVs in Brazil			
1961	Cotia county in Rio de Janeiro state	Cotia virus from mouse brains. Confirmed as poxvirus in 1978. The virus has been re-isolated consistently and proposed to be a recombinant between leporipoxviruses and OPXVs	[22, 41]
1963	Belem-do-Para, Brazil	The BAV was isolated in 1963 from blood of an <i>Oryzomys</i> rodent in the tropical rain forests surrounding Belem-do-Para, Brazil. Fonseca et al. [16] proposed to include this virus as a member of the <i>Poxviridae</i> family and suggested that it is a vaccinia variant	[16]
1979	Cotia county, Brazil	SPANv was isolated from sentinel mice that had been exposed in the Cotia forest. This virus was characterized as VLVs	[8]
1999	Cantagalo, Santo Antonio de Padua, Miracena etc. in North-western part of Rio de Janeiro state	CTGV was isolated from specimens received from an outbreak of exanthema, resembling cowpox, in dairy cattle in several farms in many counties of Rio de Janeiro state. Disease was also transmitted to milkers and their families who showed lesions on the hands and/or forearms	[9]
2001	Brazil Pariba valley region in Sao Paulo and Minas Gerais states; Sao Patricio valley in Goias State, Brazil	VBH was isolated from clinical specimens from a mousepox-like outbreak at the Biological Institute of the University of Minas Gerais, Brazil. Mice were procured from the University of Campinas, State of Sao Paulo, Brazil. This virus was characterized as VLVs	[40]
Human VLVs			

Fig. 1 World-wide distribution of Vaccinia-Like Viruses in Brazil (purple), India (red), Nepal (pink), Pakistan (green), Egypt (yellow) and Indonesia (brick red). (Color figure online)

[45] (Fig. 2b). Further, human cases of buffalopox were investigated at Aurangabad during 2008–2009 and recently near Pune, Maharashtra in 2009 and the etiology were confirmed as caused by BPXV [4]. Zoonotic infections due to different VLVs are described [44] in Table 1.

Analyses of BPXV gene sequences namely H3L, A27L, D8L, H4L, ATI, HA, B5R and C18L homologues of VACV using isolates of cows and buffaloes have been carried out to elucidate their genetic relationship to VACV and other OPXVs. The nucleotide (nt) and the deduced amino acid (aa) sequences revealed high sequence identity of BPXV isolates with VACV ($\approx 99\%$ sequence identity) both at nt and aa levels than other OPXVs. However, C18L gene based analysis showed BPXV isolates cluster into a separate group and is distinct from VACV. A number of BPXV isolates associated with outbreaks both in cows and buffaloes revealed that pox-like disease in cows is caused mostly by BPXV rather than CPXV indicating the circulation of mostly BPXV in Indian cows and warrants the

implementation of control measures [45]. Transmissibility of BPXV between different species including cows, buffaloes and human beings implies the potential reemergence of the virus in the subcontinent much similar to the VLV outbreaks witnessed recently in other countries. Based on the sequence information of these genes, BPXVs isolated from various geographical regions of India are phylogenetically very closely related to VACV (vaccine strains) than to OPXVs [34, 36]. However, genome based phylogeny would provide more conclusive evidence on the phylogeny of BPXV isolates in light of the previous observations that the virus is a clade of VACV [14].

Over the recent years, emergence of human and animal pox infections caused by VLVs have been reported from several parts of the world in Brazil. Further, cowpox disease has been reported from EU nations like Germany, Norway and Sweden, zoo-kept elephants in Germany since 1961. Seven amplicons of HA gene (five from cows and two from buffaloes) from pox virus isolates were cloned

Fig. 2 a Phylogenetic analysis based on the deduced aa sequence of hemagglutinin (HA) gene. a Vaccinia and Vaccinia-Like Viruses (b) members of Orthopoxviruses. Un-rooted tree was constructed using neighbor-joining method of MEGA version 4. Numbers on the tree branches represent the bootstrap support calculated per 1,000 bootstrap replicates. The scale bar represents the aa substitutions per site. BPXV isolates BPXV 2-04C (Cow); BPXV 9-04C (Cow); BPXV 17-04C (Cow); BPXV 13-03C (Cow); BPXV 14-03C (Cow); BPXV 1-04B (Buffalo); BPXV 18-04B (Buffalo) analyzed with various species/strains of various members of OPXVs are given;

vaccinia virus (VACV) strain-WR, strain-Lister (Li), strain-Modified Vaccinia Ankara (MVA), strain-Acambis clone 3 (Aca), strain-Tian Tan (TT), VAVC-Copenhagen, VAVC-Duke, VAVC-Ankara, Rabbitpox virus (RPV or RPXV), strain-U23 (U23), CPXV strain-GRI90, CPXV-strain Brighton Red (BR), ARAV, MPXV strain-Zaire 79 (ZR), strain-Walter Reed (WR), Camelpox virus (CMLV) strain-CMS, strain-M96, Variola virus (VaV) strain-Garcia 1966 (GAR66), strain-Bangladesh 1975 (BAN75), strain-India 1967 (IND67). BAV, *Belo Horizonte* virus (VBH), Guarani P1 virus (GP1V), Guarani P2 virus (GP2V), Passatempo virus (PSTV), CTGV

and their sequences showed 945 bp ORF encoding a polypeptide of 314 aa. At the nt level, BPXV isolates from cows and buffaloes shared 98.8–100 % sequence identity among themselves and 96.2–99 % sequence identity with VACV (highest VACV-MVA, Modified Vaccinia Ankara). Similarly, at the aa level, they shared 97.5–100 % sequence identity among themselves while with VACV, it ranged from 94.6 % (VACV-TT) to 98.7 % (VACV-MVA). Among all the OPXVs, the least sequence identity of BPXV was observed with VARV major, including VARV IND67 and VARV BAN75 followed by VARV minor (VARV-GAR66) at both nt and aa levels [45].

Multiple alignment of the aa sequences showed varying ORF among OPVs. The HA gene of the Indian BPXV isolates encoded polypeptide of 314 aa, like in CPXV and VACV with exception of VACV-MVA, the latter being 315 aa long. The gene is smallest (308 aa) in the Brazilian ARAV and longest (319 aa) in the camelpox virus (CMLV) isolate CMLV-M96. Among all the BPXV isolates, BPXV1 14-03 showed higher divergence at the aa level with substitutions like V64I, L67F, D82N, H145Y, I223V, S231P. In comparison to the aa sequence of VACV, the viruses showed four important substitutions including D213Y, V223I, N253D and Y301C. Phylogenetic analysis of BPXV isolates based on full-length HA gene showed very close relationship among themselves, forming sub cluster with VACV (Fig. 2a). Further, the BPXV isolates were more closely related with VACV than the Brazilian ARAV to VACV. This was also consistent with deletions of six codons (T, Y, N, D, D and D) in the ARAV in comparison to the aa sequence in the HA gene of either VACV or BPXV isolates. Similarly, outbreaks of disease in cows and/or humans in Brazil caused by ARAV, CTGV, Passtempo virus, Guarani P1 and P2 have been reported to be closely related to VACV based on the sequencing and phylogenetic studies. ARAV showed 95.7–96.4 % identity with VACV, which is less than the sequence identity of the BPXV isolates observed with VACV strains.

Sequence and Phylogenetic Analyses Studies on BPXV

Sequence analysis based on the nt and aa sequences of the viruses provide a better understanding of the evolutionary relationships among the closely related virus species. To determine the relationship of BPXV with other member species of OPXV, a number of BPXV genes that play a vital role either in pathogenesis or morphogenesis as established in the VACV model have been sequenced [32]. These include major envelope genes of BPXV homologues of VACV genes viz. envelope proteins that bind cellular heparan sulfate (H3L and A27L) and chondroitin sulfate (D8L), which play prime role in VACV attachment and

cell entry [21]. Nucleic acid sequences of selected genes of BPXV isolates showed >99 % sequence identity not only among themselves but also with VACV. Many substitutions in the genes that were unique to both BPXV and VACV among all OPXV were identified. Phylogenetic analysis based on structural protein genes including H3L, A27L, D8L [32] and B5R [34] and non structural protein (H4L) homologue genes [35], that are highly conserved across the genus, showed that BPXV is phylogenetically closely related to VACV vaccine strains.

Further, analysis of the sequence of the BPXV homologue of the C18L gene of VACV revealed marked changes in relation to the corresponding VACV sequence [36]. PCR amplicons corresponding to VACV complete ORF generated from six BPXV isolates revealed that the gene is disrupted in the latter, implying that BPXV lacks a functional gene homologue. In this context, a conventional gel based diagnostic PCR of uniplex and duplex format based on this gene has been developed to differentiate BPXV from other OPXV, and particularly very closely related with VACV. In a similar way, a BPXV specific fluorogenic hydrolysis probe based real time PCR using this C18L gene has been developed for rapid and sensitive detection of these viral DNA from suspected clinical samples [36]. Further the sequence and phylogenetic analysis based on partial C18L gene sequence of BPXV isolates of different host species namely buffaloes, cows and human from India revealed that the BPXV is distinct from other OPXVs (Fig. 3) and very closely related to VACVs [4, 45].

Fig. 3 Phylogenetic analysis of Buffalopox virus (BPXV) isolates from India and other members of OPXVs based on deduced aa sequence of C18L gene. Details are same as mentioned for Fig. 2

VLV Outbreaks in Brazil

The literature shows that the occurrence of human VLV outbreaks is rare. However, occurrence of human infection caused by VLV has been reported recently from Brazil [25] and India [35]. They reported that analysis of 74 human samples received from these states of Brazil during 2001–2003 suggests involvement of humans. Also at the same time, there was a great “bovine variola” outbreak that affected 1,500 cattle (*Variola bovina*, 2001; 2002; 2003). Outbreaks of BPXV (another VLV strain) have been described in India. Nagasse-Sugahara et al. [25] concur with Damaso et al. [9] that the viruses detected in Brazil could also be a new VLV strain, epidemiologically similar to BPXV. However, it is not known precisely for how long these viruses circulated in Brazil but there are reports on “bovine variola” or “vaccinia” occurrences in humans, since 1910 [29]. Nagasse-Sugahara et al. [25] further report that several old articles describe outbreaks with clinical and epidemiological characteristics similar to those that are occurring now.

Aracatuba Virus

Trandade et al. [39] recently reported isolation and characterization of ARAV from cowpox-like cases affecting milking cows and milkers, in Sao Paulo, Brazil. This virus could be easily identified as poxvirus by pock morphology on CAM of embryonated chicken eggs (ECEs). Sequence analysis of vaccinia growth factor (*vgf*), thymidine kinase (*tk*) and hemagglutinin (*HA*) genes of ARAV revealed (i) the *vgf* and *tk* genes of this virus share 99.5 % nt similarity with that of VACV-WR (Western Reserve), (ii) HA nt sequence contained a signature deletion (18 nts) identical to a deletion detected in the sequence of the CTGV [39]. Damaso et al. [9], while comparing CTGV with VACV-IOC HA sequence, reported that the HA sequences of VACV-IOC were like CTGV in that they lacked “DTYNDN” aa sequences. The results of the gene sequence analysis of ARAV, CTGV and VACV-IOC led to conclude that (i) the ARAV and CTGV-very closely related VLVs are circulating in Brazil, (ii) the ARAV could be another VLV or could represent the spread of CTGVs, (iii) the ARAV and CTGV might have been derived from VACV-IOC, the Brazilian smallpox vaccine strain.

Cantagalo virus

CTGV was isolated from cows and milkers in an exanthema outbreak during 1999 from farms in Rio de Janeiro, Brazil [9]. The molecular analysis of the HA and A-type inclusion (ATI) genes by PCR–RFLP, HA gene sequence analysis, ATI protein western blot analysis of the CTGV

and other VACV strains including vaccine strains (VACV-IOC, VACV-NYBH Wyeth, VACV-WR) revealed that the CTGV and VACV-IOC are as similar and as different as one strain of VACV is to another [9]. Further, CTGV introduction into nature remains a matter of debate. The comparison of the HA gene sequence of CTGV and other VACVs indicated that CTGV is very closely related to VV-IOC followed by VACV-Lenny, VACV-Lister, VV-Copenhagen, VACV-koppe, recombinant RPXVu23, RPXV-Utrecht, VACV-ihdj, VACV-TT and VACV-WR. The CTGV HA sequence has a signature that is the absence of six aa (DTYNDN, positions 251–256), which are present in other OPXVs examined, with the exception of RPXV, which lacked 2 of the 6 aa (ND) and VARV major, which showed Y to H substitution in these sequences. Thus, the HA aa sequence of VACV-IOC and CTGV were similar in that they lacked the DTYNDN sequences and the nt and aa similarity between the CTGV and VACV-IOC were 98.2 and 96.7 %, respectively. Further, the HA gene sequence of virus isolates from cattle and human outbreaks occurred simultaneously in Santo Antonio de Padua County, RJ, Brazil revealed 100 % identity indicating that the human infection was probably caused by CTGV. All the molecular data supported the hypothesis that CTGV is a descendent of Brazilian smallpox vaccine strain, VACV-IOC, which was used as smallpox vaccine in Brazil during 1960–1970 [9].

As is evident from HA gene sequence comparison and phylogeny of CTGV and many VACV strains that VACV-Lenny, VACV-Lister and VACV-Copenhagen were the nearest neighbours to CTGV and VACV-IOC. The possible origin of CTGV from VACV-IOC gets further support from the fact that VACV-Lenny was isolated from a Nigerian woman who developed an illness similar to eczema vaccinatum [5], and VACV-Lenny resembles the VACV-NYBH Wyeth vaccine strain which was used for vaccination at that time in Nigeria [14]. Although the HA gene sequence could establish that CTGV may derive from Brazilian smallpox vaccine strain VACV-IOC, the DNA restriction analysis indicated a lot of nt polymorphisms between these two viruses. Similar differences could exist between other VLVs (BPXV, ARAV, VBH, SPAn-232 and BAU and Cotia virus) and their progenitors such as VACV strains used as smallpox vaccine in that region. As such, extensive genome DNA sequencing is essential to fully unravel the nt differences between the two closely related viruses as well as to resolve the genetic relation of VLVs and their progenitors.

Belo Horizonte Virus

VBH was isolated from clinical specimens of mousepox-like outbreak in Brazil after passaging in CAM of ECE

[10]. Molecular characterization of VBH by DNA cross hybridization using VACV DNA, PCR-RFLP, and genome sequencing of genes viz. *tk*, *HA*, and *vgf* revealed that VBH is very closely related to VACV. *Hind*III restriction profile and nt sequence homology identical to VACV-WR. The *tk* and *vgf* genes of VBH > 99 % identical that of VACV-WR, while, *HA* gene showed only 95 % homology. Further, the similarity between VBH and ECTV Moscow was lower, reaching 96, 90, and 93 % for *tk*, *vgf* and *HA* genes, respectively. As such, the VBH genes clustered with VACV genes in all phenograms and, thus, are more closely related to VACV than VARV [40]. The VBH genome contains only 112 nt of A-type inclusion (*ATI*) gene encoding only the C-terminal portion of the ATI protein as was revealed by nt sequencing [40]. Other VACV strains also lack the *ATI* gene [26].

SPAn-232 and BAV

Two strains of poxvirus, SPAn-232 and BAV have been isolated from wild rodents from Brazil during 1960s [19]. The BAV, a naturally attenuated wild OPV isolated from the blood of *Oryzomys* wild rodents in a tropical rain forest surrounding areas of Belem-do-Para, Brazil. This virus was found to be antigenically related to the Cotia virus, a poxvirus also isolated in Brazil. BAV was initially classified in Arbovirus and remained uncharacterized for some time. Later, the characterization of the virus proved that it should be included in the *Orthopoxvirus* genus [16]. Sequence analysis of BAV *tk* and *IFN- α/β R* genes (a homologue of B18R in VACV-WR) showed 99 % homology for both the genes with VACV-WR strain as was also evident in phylogenetic trees constructed based on the sequences of *tk* and *IFN- α/β R* genes of BAV and VACV, VARV, CPXV wherein BAV always clustered with VACV-WR [23]. The *IFN- α/β R* gene identified in BAV genome encodes a soluble receptor for type I IFN (*IFN- α/β R*) responsible for the IFN inhibitory activity as observed with VVWR B18R gene. This also confirms that BAV is a variant of the VACV vaccine strain used in Brazil [23]. Further, the future perspectives for BAV include: (i) explanation for the lack of virulence in BAV, and (ii) effect of lack of ATI in BAV, as that of VACV. In view of the above, it can be presumed that BAV is possibly a natural attenuated VACV sample (may be derived from VACV-Lister the smallpox vaccine strain used in the region from where BAV was isolated) that was in circulation for many years in forest regions of Brazil. However, this presumption contradictory because BAV has a functional *IFN- α/β R* gene but lacks ATI homologue but, conversely, VACV-Lister lacks *IFN- α/β R* gene but encodes ATI gene.

SPAn-232

The virus SPAn-232, isolated from a sentinel mouse in the rural areas of Cotia-SP [19], characterized as a VLV [8]. Nagasse-Sugahara et al. [25] apprehend that, SPAn-232 could be related to all the VLV that are detected in Brazil. Even though the vector of this virus is unknown virus has been isolated from a sentinel mouse, which suggests that vector could be an arthropod [8]. The comparison of the sequence of these two isolates with VLVs isolated recently in Brazil may give insight in molecular epidemiology of the VLVs. Similarly, seroepidemiology would also be important to evaluate the prevalence of specific antibodies in the population. The antibody levels in human, cattle and wild animals would help to understand the mechanisms of infection as well as evaluation of population's protection level against VARV, a biological warfare agent against human population [25].

Production of Vaccine in Animals Vis-à-Vis Spill Over and Spill Back

Historically, many strains of VACV (Lister Elstree, Dryvax (New York City Board of Health, NYCBH strain), CV-1, EM63, MVA, NYVAC, LC16m8, Wyeth and NYCBH) had been used as smallpox vaccine. In the intensified WHO global eradication campaign, these were largely replaced with two strains, the Elstree or Lister strain, and the NYCBH or Wyeth (Dryvax) strain [27]. These two strains are still available as vaccine reserves maintained as long-term freeze-dried stocks by the WHO and various national governments [27]. By the end of the nineteenth century, arm-to-arm vaccination had been made illegal in many countries and smallpox vaccine was obtained from animal skin. For historical reasons, calves were first used for vaccine production. During the First World War, the Lister Institute of Preventive Medicine, in Elstree, Hertfordshire, England, introduced the use of sheep, a practice subsequently adopted in some other countries. Because of their ready availability, water buffaloes were used in India, IndoChina, and Indonesia. They are considered to be superior to other species for smallpox vaccine production [7]. Thus, most of the smallpox vaccine viruses were grown in animals by alternate passaging in rabbits and cow or buffalo. The vaccine virus produced in calf lymph was applied as vaccine by skin scarification.

In the last century, several institutions manufactured smallpox vaccine in alternate species to avoid attenuation of the vaccine virus through adaptation to a single host [27]. It is likely that passage through multiple species acted to maintain certain level of diversity (or heterogeneity) within a vaccine population. Attempts were made in

several countries to produce smallpox vaccine in chick embryos and in cultures cells, to avoid bacterial contamination that was inevitable when production was carried out by scarification of the skin of large animals. However, chick embryo vaccines were produced on a commercial scale only in Brazil, Sweden and the State of Texas in USA, and only in Brazil there was a widespread use in an eradication campaign. Production of new vaccine stocks utilizes cell culture (in vitro) production system rather than animals (in vivo) production system as utilized in the previous century, and it is possible that the cell culture production system may lead to attenuation in the course of time, especially as it is likely that heterogeneous vaccine virus populations will quickly adapt to grow in tissue culture conditions [27]. During mass vaccination campaigns, VACV infections were occasionally transmitted from the vesicular lesion on the Vaccinee to domestic animals, usually cattle and, in turn, infected animals transmit VACV to susceptible people. Several outbreaks in cattle and humans of what was thought to be cowpox were indeed caused by VACV [38]. Of late, we established that pox-like disease in cattle was caused in most cases by BPXV rather than CPXV [45], as was thought earlier based on genetic analyses.

Origin of VLVs in India and Brazil

Based on the signature deletion in HA gene sequence of ARAV, CTGV and VACV-IOC as well as >99 % nt similarity between *tk* and *vgf* genes of ARAV and CTGV with VACV strains, it is tempting to speculate that the ARAV and CTGV might have evolved from VACV-IOC, the Brazilian smallpox vaccine strain. A number of buffalopox outbreaks have been reported from different states of India from time-to-time since 1934. Recently, our laboratory reported an outbreak from Aurangabad, Maharashtra, which was associated with high morbidity and productivity loss in the buffalo dairy herds including zoonotic infection in humans [33]. Subsequently, the disease that occurred in Nellore, Andhra Pradesh during April and August, 2006 also caused high morbidity in the dairy animals [4]. Many of such outbreaks although infected primarily buffaloes, cows were also involved in mixed flocks apart from zoonosis. Analysis of these isolates, based on full-length gene sequence, proved that aetiological agent of pox disease in both cows and buffaloes was BPXV [44]. Circulation of BPXV in cows apart from milch buffalo and humans as evident from the present investigation is a serious public health concern. In recent times, BPXV has been implicated in severe human-to-human transmission as seen in a hospital in Karachi, Pakistan, where spread of infection across several burn units was noticed. Under

these circumstances, transmissibility of the virus from humans to animals and vice versa cannot be ruled out. Frequent epidemics of the buffalopox infection in certain parts of the country involving domestic animals particularly buffaloes and occasionally cows hint at possible involvement of the wild reservoir host which cannot be ruled out in the present circumstances.

Similarly, molecular characterization of various VLV viruses isolated from Brazil and India suggests association of these viruses with VACV vaccine stocks that may have escaped to wild when the vaccination program was in operation during 1970–1980. However, pin-pointing the exact origin of these viruses is difficult since many different vaccine virus strains were employed in smallpox vaccination programs in the world. On an enquiry by WHO from 59 vaccine producing organizations throughout the world, 23 employed the Lister strain, 7 the NYCBH strain, and the 7 Paris strain while the remaining 22 laboratories used a number of strains, none of which was used by more than 3 laboratories (Table 2). Further, the Lister strain was the most widely used VACV strain for smallpox vaccine production in Asia and Oceania [14]. India and other neighboring countries mostly used Lister strain except USSR where B-51 and EM-63 strains were also used besides Lister strain from where India imported smallpox vaccine. The VACV strains viz. VACV-Lister, VACV-IOC, VACV-WR was employed in Brazil [39]. Nonetheless, outbreaks of buffalopox in India, Pakistan and Brazil (ARAVs/CTGVs/Passtempo/Guarani P1 and P2 viruses) in the recent years underscore the reemergence of the VLVs in these regions.

Vaccine Strain Vis-à-Vis Escape of Vaccine Virus

Size of skin lesions depends on strain of vaccine virus used for immunization. All the live modified vaccines induce “vaccine-take” (pustules, scabs and scarring). It takes a fortnight after immunization for the lesions to attain maximum size. Further, the smallpox vaccine viruses were heterogenous population, as the seed viruses used for vaccine production in animals were not clonally purified. There have been no detailed studies on many vaccine strains used for immunization and the size of skin lesions. Presumably, however, different vaccine viruses might have produced pock lesions of varying sizes as had been evident from the extent of post-vaccinal complications induced by different smallpox vaccine viruses. For example, VACV (Copenhagen) induced much more post-vaccinal encephalopathy in vaccinated infants of <1 year age than Lister strain (37.7 cases of encephalopathy per million by Copenhagen strain as against 11.1 cases of encephalopathy per million by Lister strain). Recently, dynamics of skin

Table 2 Vaccinia virus (VACV) strains used in smallpox mass immunization program

Sl. no.	Country	Strain used during the year	
		1968	1971
Asian and Oceania continent			
1.	India	Lister, Patwadangar	Lister, Patwadangar
2.	Pakistan	Not known	Lister
3.	Burma	Lister	Lister
4.	Indonesia	Lister	Lister
5.	China	Temple of Heaven	Temple of Heaven
6.	China (province of Taiwan	Lister	Lister
Africa			
1.	Egypt	Not known	Lister
Americas (Brazil and neighbouring countries)			
1.	Brazil	Paris, New York, Lister	New York, Lister, VACV-IOC ^a , VACV-WR ^b , mixtures of different samples ^a
2.	Canada	Lister	Lister
3.	USA	New York	New York
4.	Ecuador	Not known	Lister
5.	Peru	Not known	Lister
6.	Columbia	Lister	Lister
Countries from where India imported smallpox vaccine			
1.	USSR	B-51, Tashkent, EM-63	B-51, LE-IVP (Lister), EM-63
2.	United Kingdom	Lister	Lister
3.	Netherlands	Lister	Lister
4.	Switzerland	Lister	Lister

Modified from Fenner et al. [13, p. 584] and supplemented from Trandade et al. [39] and Damaso et al. [9]. India also imported smallpox vaccine from USSR, Netherlands, Switzerland and United Kingdom

^a Vaccinia virus—Instituto Oswaldo Cruz

^b Vaccinia virus—Western Reserve

lesion progression was studied for two VAVC vaccine strains viz. Lister and LC16m8, wherein, it was observed that, on day 13 post immunization, lesions produced by LC16m8 and Lister strains were of 27 ± 11 and $115 \pm 65 \text{ mm}^2$, respectively. Thus, the lesions produced by Lister strain were much larger, exudative and granulomatous in comparison to that produced by LC16m8 strain. Satellite lesions appeared with Lister but not with LC16m8. Similarly, on day 28, the pigmentation of the scars was apparent with Lister, but not with LC16m8 [28]. These studies indicated that LC16m8, a highly attenuated virus, would be much safer with minimum escape.

Zoonosis

As is evident from the foregoing discussion that vaccinia-like cases from human, cows and buffaloes have been recorded in India and Brazil regularly but there are only limited studies about molecular characterization of poxviruses in human, cows and buffaloes throughout the world. This points the need of improving the epidemiological surveillance with a seroepidemiological study that would help to determine the transmission chain, and to identify the natural host of these viruses as this zoonosis is

detrimental to the local economy besides being a global public health concern. All the viruses discussed, so far have potential to cause concurrent pox-like disease in cattle, buffalo and humans.

Establishment of VLVs in Nature

CPXV is found throughout Europe and western Asia [2]. In Great Britain, antibody has been found occasionally in house mice but the highest seroprevalence is in bank voles, wood mice, and field voles, and these species are believed to be the reservoir hosts [6]. Cowpox does not cause obvious clinical signs or increase mortalities in voles or mice in the laboratory [3], experimental studies have demonstrated that cowpox can affect fecundity by delaying the onset of reproduction [15]. Further, it is thought that the CPXV infection, due to many reasons, might have greater impact on survival in field conditions [37] which might further help in establishment of CPXV infection in these wild rodents. Reservoirs for VACV or VLVs have not been established. Even though, the reservoirs (wild rodents) for CPXV have been reported in Europe and adjacent parts of Asia, there are no reports in the published literature about isolation of BPXV from other animal species except

buffaloes and cows and therefore reservoirs for BPXV are yet to be established in India. However, two viruses viz. BAV [16] and SPANv [22] have been isolated in Brazil from sentinel mouse and wild rodent, respectively, but not as reservoirs for these VLVs. Establishment of VLVs might have occurred because of the (i) adaptation of VACV vaccine virus to host in which it was passaged like buffaloes in India, Egypt, Bangladesh, Pakistan, Nepal and in cattle in Brazil and (ii) contact of Vaccinee with domestic and pet animals resulting in cross-contamination from open wounds caused by intra-dermal (i/d) immunization. Spill over of smallpox vaccine virus from Vaccinee to in contact humans has been reported.

Establishment of VLVs is considered to have occurred because of the (i) adaptation of VACV vaccine virus to host in which it was passaged like buffaloes in India, and (ii) contact of Vaccinee with domestic and pet animals resulting in cross-contamination from open wounds upon i/d immunization. The sub-cutaneous (s/c) route of poxvirus inoculation has been reported to be effective in pathogenesis and for elucidation of immune response in mice. Further, s/c route of immunization of goats with goatpox vaccine [18] and camels with camelpox vaccine [17] were equally effective as i/d route in terms of eliciting immune response. Thus, it seems plausible that s/c route can be used for smallpox vaccination in human but needs a plenty of data before it is executed. In future, the smallpox vaccine will be produced in vitro, which will totally prevent the use in vivo cultivation of vaccine virus. Thus, use of in vivo system for smallpox vaccine production and s/c route of immunization will significantly minimize spill over of vaccine virus from Vaccinee to incontact human/pet/domestic animals.

Conclusions and Future Perspectives

Since the cessation of smallpox vaccination campaign owing its eradication throughout the world, the naïve population after 1980 are continuously susceptible to different OPXVs and responsible for recent emerging and reemerging form of viral infections particularly VLVs namely buffalopox in Southeast Asia and ARAV and Contagalo viruses from Brazil. This changing pattern of viral infections might be due to adaptation of VACV vaccine strain in animals either through passing the virus or close contact between pet animals and vaccinated humans. It may be speculated that the BPXV isolates recovered from human in India would have been gradually adapted in human due to the close contact of human with the affected animals or could be due to circulating VACV in nature. Sequence analysis of immunogenic structural protein genes, which aid in the attachment, virulence and

pathogenesis, may be of immense value to abreast the knowledge on BPXV of human origin. In similar way, other VLVs in other parts of the world have to be studied in detail. This warrants a detailed systematic study on virus epidemiology, existence of reservoir host namely rodents, biological transmission, and molecular organization of different VLVs may pave the way for control of the diseases by prophylactic measures.

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