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An outbreak of buffalopox in buffalo (*Bubalus bubalis*) dairy herds in Aurangabad, India

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Summary
An outbreak of buffalopox in domestic buffaloes, with high morbidity and significant production loss, was recorded in the Aurangabad district of Maharashtra State in India in November 2003. The disease was also associated with several cases of human infection, particularly in milkers working with the affected herds. Pox lesions were observed on the udder and teats of the majority of the affected animals, while a few animals exhibited lesions on the hindquarters, indicating possible generalised infection. A significant reduction in milk yield was recorded following the outbreak. Milkers developed pox-like lesions on the skin of the hands, forearms and forehead accompanied by fever for three days, axillary lymphadenopathy and general malaise. Investigation of the disease outbreak by virus isolation in Vero cell cultures and detection of viral nucleotide sequences by polymerase chain reaction (PCR) confirmed the aetiology of the disease.

Keywords
Buffalopox virus – Indian Buffalo – Polymerase chain reaction – Zoonosis.

Introduction

Buffalopox is an important zoonosis of domestic buffaloes (*Bubalus bubalis*) associated with high morbidity and productivity losses. Disease outbreaks often lead to considerable reduction in the milk yield of affected dairy herds in addition to a reduction in the working capacity of infected animals. It has been estimated that production of buffalo milk in the Asia-Pacific region exceeds 45 million tons annually, of which 30 million originate from India (5). Thus, the impact of this disease on the dairy industry and economy in the region is highly significant. Buffalopox is also recognised as an important zoonotic infection (9). People born after 1977 are potentially susceptible to infection with buffalopox virus, a close variant of vaccinia virus (8, 16), because smallpox vaccination ceased worldwide in 1980.

Buffalopox is prevalent throughout the major buffalo-rearing areas of the world, and outbreaks have been reported in many countries including Indonesia, Egypt and Pakistan (12). The disease has been recorded since 1934 in

different parts of India (2, 3, 7, 11, 14, 17, 19) and in the last decade outbreaks of buffalopox have been reported from many states including Uttar Pradesh, Rajasthan, Andhra Pradesh, Gujarat and Karnataka (unpublished data). The disease is caused by buffalopox virus (BPXV), a prototype member of the *Orthopoxvirus* (OPV) genus in the family *Poxviridae*. In this paper, the authors report an outbreak of buffalopox in dairy herds on the outskirts of Aurangabad which caused high morbidity and production losses in the affected herds and also zoonotic infections in animal handlers and milkers.

Disease outbreak and clinical picture

The outbreak of buffalopox occurred in November 2003 on the outskirts of the Aurangabad district of Maharashtra in India. The outbreak occurred in ten herds containing buffaloes of mixed ages and of predominantly the Jafarabadi breed and the Jafarabadi × Surti crossbreed of domestic buffalo. The farms were individually owned with a total population of animals at risk of approximately 400. The overall morbidity reached 45% (a total of 180 of the 400 buffaloes). Approximately 80% of the affected buffaloes (which were aged between 6 and 12 years) were Jafarabadi and Jafarabadi × Surti dairy animals. The exact number of buffalo bulls affected in the outbreak could not be ascertained. However, two buffalo bulls intended for breeding were found to be mildly affected in one of the herds under investigation; the lesions were confined to the hindquarters in these cases.

Clinical signs, such as lesions on the udder, teats and hindquarters of the affected animals, were suggestive of pox infection (Fig. 1). Characteristic circumscribed ulcerated lesions with raised edges were observed, which were painful on palpation. About 40% to 50% of the affected animals showed mastitis and reduced milk yield, mainly due to secondary bacterial infections. However, four cases of mastitis and stricture of the teat canal directly attributable to pox lesions were observed and permanent loss of milk production as a consequence of mastitis was reported in one case.

The exact source of the infection could not be ascertained. However animal trade between villages (Dhulia and Chauvi Bazar) was evident from the history, and this may have contributed to the spread of the infection. The outbreak started a month before the survey began, and only three to four herds showed fresh clinical cases of disease at the time of investigation.

Materials and methods

Collection and preparation of clinical materials

Scabs from pox lesions were collected primarily from the thigh region, udder and teats of affected buffaloes. Blood and milk samples were also collected from affected animals, and from in-contact animals with no apparent lesions, for serodiagnosis. The scab materials were ground with a pestle in a mortar containing sterile sand. Phosphate buffered saline (PBS) was added to prepare a 10% (w/v) suspension. The samples were stored at –20°C until further use, either for the extraction of DNA or for the isolation of virus in cell culture.

Counterimmunoelectrophoresis

Preliminary screening of the scab and serum samples was carried out using counterimmunoelectrophoresis (CIE)

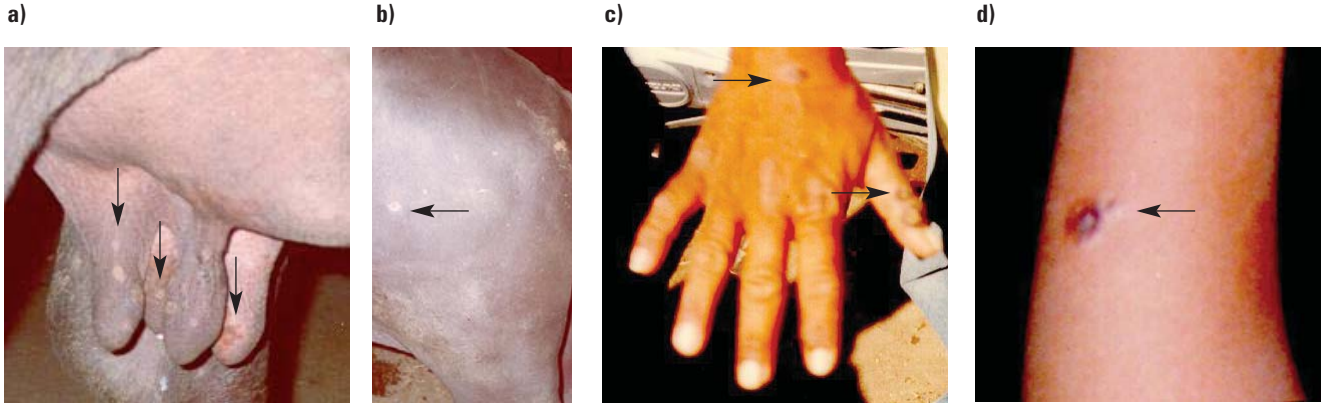

Fig. 1
Clinical lesions in buffaloes and milkers infected with buffalopox virus
Typical pox lesions found on the udder/teats of milk buffalo (Fig. 1a) and pre-inguinal region (Fig. 1b) of one of the affected buffalo bulls, which was used for breeding purposes. The disease was also associated with human infections, with typical pox lesions noticed on the fingers (Fig. 1c) and forearm (Fig. 1d) of milkers

(18) with a reference antigen (BPXV-BP4) and its hyperimmune serum (HIS). The BPXV antigen was derived from infected Vero cells by clarification of the infected cell culture fluid then concentrated by precipitating the viral antigen with 8% (w/v) polyethylene glycol (PEG 6000).

Virus isolation

For virus isolation, scab samples were ground in sterile PBS to make a 10% (w/v) suspension. The tissue triturate was repeatedly frozen (at -80°C) and thawed 2 to 3 times and then subjected to ultrasonication for 2 min at 30% amplitude in pulse bursts of 9 s duration with 9 s intervals to extrude virus particles from the cells and inhibit bacterial and fungal contamination. Following clarification at 6,000 × g for 2 min in a microcentrifuge (Sigma 1-13, Germany) 0.3 to 0.4 ml of the supernatant was treated with gentamicin at a concentration of 40 µg/ml (Gentamicin Injection, Cadilla Pharmaceuticals, Ahmedabad, India) in Eagle's Minimum Essential Medium (EMEM) and inoculated onto Vero cell monolayers. Ultrasonicated milk samples were similarly inoculated onto Vero cell monolayers. Cells were incubated at 37°C for one hour with intermittent shaking to allow adsorption of virus. The virus inoculum was then decanted and the infected cells were washed 4 to 5 times with serum-free EMEM. Finally, the infected cells were fed with maintenance medium containing 2% foetal calf serum and incubated again at 37°C. Flasks were observed daily for the appearance of cytopathic effect (CPE).

Viral deoxyribonucleic acid preparation

Total viral DNA was extracted either from scab material or from infected cell culture fluid using the AuPreP™ DNA Extraction Kit (Life Technologies (India) Pvt Ltd, New Delhi, India) following the manufacturer's protocol. DNA was extracted from a 200 µl initial volume of either scab supernatant or infected cell culture fluid and finally eluted in 50 µl of nuclease-free water for PCR.

Polymerase chain reaction

Viral DNA isolated from clinical material or cell culture fluid was subjected to PCR for amplification of a fragment of the A-type inclusion gene homologue (ATI) using the following primer pair: CoPV03 (GGGATATCAAGGAAT GCGA) and CoPV04 (TCCATATCAGCATTGCTTTC). The full-length inclusion gene was amplified using the CoPV01 (TAAATGGAGGTCACGAAACT) and CoPV02 (TTCCCA TTCACGTTCGCATG) primer pair (10, 15). For diagnostic PCR, DNA samples prepared from BPXV-BP4-infected Vero cells and uninfected Vero cells were also included in the PCR reaction as positive and negative controls, respectively. For amplification of the full-length gene, camelpox virus DNA was used as the positive control, while uninfected Vero cells were employed as the negative control. The PCR products were analysed by 1% agarose gel electrophoresis stained with ethidium bromide to visualise amplified products.

Cloning and sequencing

The PCR products were gel-eluted using a commercial kit following the manufacturer's protocol (Qiagen, Hilden, Germany) and cloned into plasmid vector pTZ57R/T (MBI Fermentas, Hanover, Maryland, USA). Recombinant plasmids purified using the SV minipreps plasmid purification system (Promega Corporation, Madison, Wisconsin, USA) were screened for the verification of the cloned insert by both PCR and restriction endonuclease digestion. The nucleotide sequence of the cloned insert was determined by the dideoxy chain termination method using an automated DNA sequencer (ABI Prism 377, Applied Biosystems, Foster City, California, USA). The sequence similarity of the gene fragment of the inclusion gene of BPXV to other OPV sequences available in the GenBank database was determined using the Clustal W program of the Lasergene 6.0 package (DNASTAR Inc., Madison, Wisconsin, USA).

Serum neutralisation test

Serum samples collected from both clinically affected and in-contact animals were subjected to a serum neutralisation test for detecting antibodies against BPXV using standard procedures (1). The serum samples (in duplicate rows) were diluted in Eagle's maintenance medium in a two-fold dilution series from 1:2 to 1:64 in flat-bottomed 96-well tissue culture plates. The reference (50 µl/well) BPXV-BP4 virus suspension with a titre of 100 $TCID_{50}$ in 50 µl was added to each serum well and the mixture was incubated for one hour at 37°C in a humidified CO_2 incubator. An aliquot of 50 µl of Vero cell suspension (approximately 1.5 × 10^6/ml) was added to each well and the plates incubated. Serum (positive and negative control sera), virus and cell controls were included in the procedure. The plates were observed for CPE for 3 to 4 days. The highest dilution of serum inhibiting the CPE of virus at the 50% endpoint was taken as the neutralising titre of the serum sample.

Results and discussion

Buffalopox is a highly contagious viral disease affecting primarily buffaloes, its natural host, and can often be transmitted to in-contact humans. The disease is economically significant through a reduction in milk

production, impaired capacity to work and lowered meat production in affected animals. Although the disease is associated with almost no mortality in buffaloes, the morbidity can be high (4, 7).

The disease occurs in epidemic form in India with several outbreaks reported in recent years (7, 11, 17). As a result of the cessation of smallpox vaccination of humans in 1980, cross-species infection with animal poxviruses such as BPXV, which is closely related to vaccinia and variola (smallpox) viruses, poses a significant public health risk. Humans with the disease develop localised pox lesions on the skin of the hands and/or forehead accompanied by fever and regional lymphadenopathy.

The outbreak reported here occurred in buffalo herds located in a small close-settled community. It was noted in the history that most of these herds had recently acquired at least one or more animals from the local markets in the Dhulia or Satara districts, which are known to be endemic for buffalopox. These introduced buffaloes were the probable source of infection for the studied herds. Disease was mostly noticed in dairy animals and the spread of disease within a given herd was probably facilitated by dairy personnel; each herd had a single milker. The owners reported that a significant reduction in the milk yield of affected animals occurred. Based on the data collected from the farmers, a 38% to 69% reduction in milk yield was recorded as a consequence of the outbreak, in addition to the permanent reduction in milk yield that occurred in some cases as a sequel to severe mastitis. The estimated cost of the veterinary treatment of one animal was Rs.400 (just over US$ 8.5) per treatment, which corresponds to a 15% to 20% loss of income per month per animal during the period of the outbreak. In milkers and other animal handlers, multiple dermal lesions on the fingers and forehead and solitary nodules on the forearm were typical of pox infection (Fig. 1). Other clinical signs observed were fever, which lasted about three days, lack of appetite, enlarged axillary lymph nodes and general malaise. Unfortunately, milkers were generally unwilling to provide biopsies and serum samples for laboratory diagnosis to confirm the disease and enable an estimate of the prevalence of this zoonotic disease in the community to be made. A number of earlier zoonotic cases of buffalopox have also been reported in the literature (11, 13, 17, 19, 21).

Preliminary laboratory diagnosis of the disease in buffaloes was carried out by CIE using HIS raised against the reference BPXV-BP4 isolate (20). Details of the samples collected and analysed by the various diagnostic tests are given in Table I. Polymerase chain reaction using OPV genus-specific ATI gene primers (CoPV03 and CoPV04) resulted in amplification of a fragment of 552 base pairs (bp), as expected (Fig. 2). Cloning and sequencing of the ATI gene fragment amplicon was carried out to confirm the fidelity of the PCR and sequence data were submitted to the NCBI GenBank database (DQ190239). The current BPXV isolate shared 99% sequence identity with vaccinia virus (VV) WR (AY243312) and rabbitpox virus (RPV) (AY484669), 97% with camelpox virus M96 (AF438165) and camelpox virus CMS (AY009089), 95% with cowpox virus GRI-90 (X94355) and 93% with cowpox virus BR (AF482758). Sequence analysis of the partial inclusion gene of BPXV (552 bp) revealed only 1% divergence from VV and RPV, clearly indicating that BPXV is a variant strain of VV. Buffalopox virus has previously been suggested to be a variant of VV, based on its host range, clinical signs, and biological and serological properties (7, 8, 16).

Table I
Analysis of clinical samples collected from the outbreak of buffalopox in Maharashtra, India, in 2003

Sample type	Number of samples giving positive results in the different diagnostic tests			Serum neutralisation titre
	Counterimmuno-electrophoresis	Polymerase chain reaction	Virus isolation	
Scabs (6)*	3/6	4/6	1/1	–
Milk (10)	ND	0/10	2/3	–
Serum (27)	2/6	–	–	14 sera tested with titres ranging from 1:2 to 1:8

* Figures in parentheses indicate the total number of samples collected
ND: not done

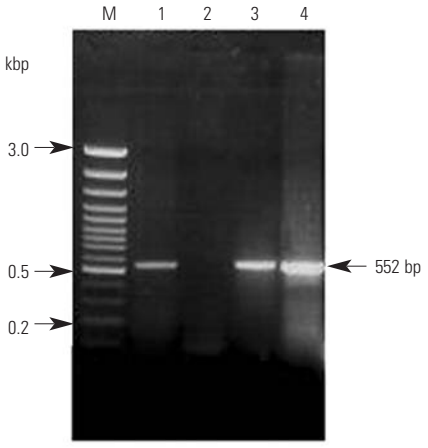

Fig. 2
Ethidium bromide-stained agarose gel electrophoresis of polymerase chain reaction products of partial gene of buffalopox virus A-type inclusion gene homologue using CoPV03 and CoPV04 primers
Lane M: 100 base pair (bp) DNA ladder
Lane 1: amplicon of 552 bp fragment from BPXV-BP4 isolate as positive control
Lane 2: negative control
Lanes 3, 4: amplicon from DNA isolated from scab material and infected cell culture fluid (passage 2 in Vero cells infected with milk samples of BPXV), respectively

Amplification of the full-length inclusion gene, using the CoPV01 and CoPV02 primer pair, was carried out for diagnosis and further genetic characterisation of the virus. The latter PCR amplifies a 3.7 kilobase (kb) product from cowpox virus (15), a 3.2 kb product from BPXV and a 2.8 kb product from camelpox samples (Fig. 3). The authors' attempts to amplify the viral nucleic acid sequences from milk failed. This could possibly be due to the presence of PCR inhibitory substances in the milk samples.

Virus isolation was successfully carried out in Vero cells using skin scab and milk samples collected from infected buffaloes. Cytopathic changes began with cell rounding and clumping on day 2 post-infection (pi), and progressed to formation of micro-plaques on days 3 to 4 pi followed by degeneration of cells and finally complete detachment of the monolayer by day 4 to 5 pi in the initial passage. Similar cytopathic changes were observed in Vero cells infected with the reference isolate of BPXV-BP4. Vero cells were demonstrated to be a suitable cell culture system for primary isolation of the virus, as cytopathic changes were appreciable as early as three days after infection of the preformed monolayer. Virus was successfully isolated from two milk samples collected from affected buffaloes. However, it required two blind passages in Vero cells to produce appreciable CPE.

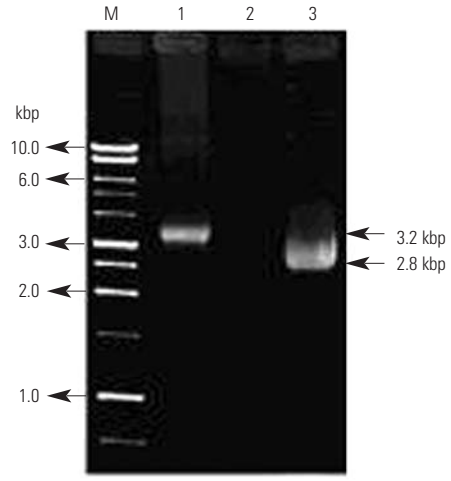

Fig. 3
Polymerase chain reaction amplification of full length A-type inclusion gene homologue of buffalopox and camelpox viruses
Lane M: 1 kilobase (kb) DNA ladder
Lane 1: amplicon corresponding to ≃ 3.2 kb from BPXV Aurangabad 2003 scab DNA
Lane 2: negative control showing no amplification
Lane 3: camelpox virus DNA used as positive control showing amplicon of ≃ 2.8 kb

PCR using template DNA extracted from both scab material and Vero cells infected with virus from milk samples yielded the expected 552 bp product (Fig. 2). This is probably the first report of isolation of virus from milk samples collected from buffaloes with pox infection. However, it is possible that the presence of the virus in the milk samples was due to cross contamination from infected teats during sampling. Regardless of the source of the virus, milk can serve as a potential vehicle of infection for other animals including suckling calves, and for milkers and personnel handling the milk. The presence of capripox virus in milk and other body fluids has been demonstrated previously, as reviewed by Davies (6).

Serum samples collected from apparently infected and in-contact animals showed neutralising titres ranging from 1:2 to 1:8 using the BPXV-BP4 isolate in Vero cells. This showed that the animals were in various stages of infection, as the outbreak was noticed in the herds one month before the incident was investigated. The outbreak reported here was severe in terms of the extent of the morbidity (up to 50%) and reports of productivity loss by farmers.

In summary, this outbreak of buffalopox was confirmed by virus isolation, serum neutralisation, PCR, and nucleic acid sequencing. Given the zoonotic importance of buffalopox infection and the high productivity losses in dairy herds, outbreaks of the disease need to be carefully monitored. Milkers, farmers and other livestock handlers should receive education on control measures such as restriction of movement of animals with lesions, basic hygiene practices and within and between herd biosecurity. This needs to be addressed to limit the spread and severity of buffalopox outbreaks and thus reduce the economic and public health impact of buffalopox on the community.

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Foyer de variole du buffle dans des élevages de bufflonnes laitières (*Bubalus bubalis*) à Aurangabad, Inde

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Résumé

Un foyer de variole du buffle s'est déclaré dans le district d'Aurangabad de l'état de Maharashtra en Inde, en novembre 2003, provoquant chez les buffles domestiques un taux de morbidité élevé ainsi qu'une baisse importante de la production. La maladie a également été associée à plusieurs cas d'infection humaine, notamment chez les ouvriers de traite. Des éruptions cutanées caractéristiques de la variole sur les mamelles et les pis ont été observées chez la plupart des bufflonnes affectées, avec quelques cas de lésions sur le train postérieur, signe d'une possible infection généralisée. Une réduction importante de la production de lait a été constatée suite à l'infection. Les symptômes constatés chez les ouvriers de traite étaient une éruption cutanée au niveau des mains, des avant-bras et du front, un accès de fièvre pendant trois jours, une lymphadénopathie axillaire et un malaise généralisé. L'étiologie de la maladie a pu être confirmée par isolement du virus sur des cellules Vero et détection des séquences nucléotidiques au moyen de la technique d'amplification en chaîne par la polymérase.

Mots-clés

Amplification en chaîne par la polymérase – Buffle d'Asie – Virus de la variole du buffle – Zoonose.

Brote de viruela del búfalo en ganados de búfalas (*Bubalus bubalis*) lecheras de Aurangabad (India)

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Resumen

En noviembre de 2003, en el distrito de Aurangabad del estado indio de Maharashtra se notificó un brote de viruela en búfalos domésticos que causó una elevada morbilidad e importantes pérdidas en la producción. Hubo asimismo varios casos de infección humana, sobre todo entre los muñidores que trabajaban con los rebaños afectados. En las ubres y tetillas de la mayoría de las hembras afectadas se observaron lesiones variólicas, y unos pocos ejemplares mostraban también lesiones en los cuartos traseros, hecho indicativo de una posible infección generalizada. Tras el brote se observó una sustancial caída en la producción de leche. Los muñidores presentaban lesiones parecidas a las variólicas en la piel de manos, antebrazos y frente, acompañadas de fiebre

durante tres días, linfadenopatía axilar y malestar general. El estudio del brote infeccioso, por aislamiento del virus en cultivos de células Vero y posterior determinación por reacción en cadena de la polimerasa de sus secuencias nucleotídicas, permitió confirmar la etiología de la enfermedad.

Palabras clave
Búfalo indio – Reacción en cadena de la polimerasa – Virus de la viruela del búfalo – Zoonosis.

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