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Outbreak of Cutaneous Papillomatosis in Buffaloes (*Bubalus bubalis*): Diagnosis by Transmission Electron Microscopy

Cappellaro, C.E.M.P.D.M[1]; Catroxo, M.H.B.[2*]; Martins, A.M.C.R.P.F.[3]

Electron Microscopy Laboratory, Research and Development Center in Animal Health, Biological Institute, São Paulo, SP, Brazil Biological Institute, Avenida Conselheiro Rodrigues Alves, 1252, Vila Mariana, São Paulo, SP, Brazil
*Corresponding Author

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Abstract— *Papillomatosis is an emerging infectious disease that causes significant economic losses in intensive and semi-intensive buffalo production systems. In dairy herds, the disease impairs milking, causes cutaneous lesions and bleeding, and predisposes animals to mastitis. In beef production systems, the main impacts include reduced weight gain, hide damage, and increased veterinary care costs. The disease may occur in cutaneous and mucosal forms. The cutaneous form is characterized by the presence of warts or fibropapillomas distributed throughout different regions of the body, whereas the mucosal form is associated with bovine enzootic hematuria and urinary bladder carcinogenesis. The lesions observed include papillomas, fibropapillomas, and fibromas, as well as verrucous lesions affecting the mucosa of the rumen, oral cavity, esophagus, and amniotic membrane. Transmission occurs mainly from animal to animal and is facilitated by injuries to the skin and mucous membranes caused by fences, water troughs, feeding troughs, or instruments used for animal restraint. Bovine papillomaviruses are double-stranded DNA viruses belonging to the family Papillomaviridae. In 1993, fibropapilloma samples from five buffaloes (Bubalus bubalis) originating from a farm located in the municipality of Registro, São Paulo State, Brazil, were submitted to the Electron Microscopy Laboratory of the Biological Institute, São Paulo, Brazil. The samples were processed using transmission electron microscopy, negative staining, and resin embedding techniques. In all samples, a large number of non-enveloped papillomavirus particles with icosahedral symmetry were observed. These particles were characterized as "full" and "empty" virions, measuring approximately 60 nm in diameter. In ultrathin sections of papilloma fragments, intracytoplasmic viral particles measuring approximately 30 nm in diameter were observed, associated with thickening of the plasma membrane, which exhibited a serrated appearance. In addition, intranuclear viral particles and membrane-bound intracytoplasmic vacuoles were identified. In the reticular layer, a nucleus with an irregular contour, marginalized chromatin, and the presence of viral particles was observed. The results confirm the occurrence of papillomatosis in buffaloes and demonstrate the importance of electron microscopy for the rapid diagnosis and morphological characterization of the viral agent.*

Keywords— Papillomatosis, Buffaloes, Transmission electron microscopy.

I. INTRODUCTION

Bovine papillomatosis is an infectious disease of viral origin caused by bovine papillomavirus (BPV). Although it is more frequently described in cattle, it also affects buffaloes, in which it may occur in both cutaneous and mucosal forms. The cutaneous form is characterized by the presence of warts or fibropapillomas distributed across various regions of the body, including the head, neck, back, ears, abdomen, gluteal region, teats, udder, vulva, and perivulvar area (Silvestre et al., 2009). These tumors, which may occur as single or multiple lesions of varying sizes, tend to develop more frequently in previously

traumatized areas, possibly due to the release of inflammatory cytokines, reactivation of latent viral infections, and stimulation of cellular proliferation (Silvestre et al., 2009; Al-Redah et al., 2021).

The mucosal form of the disease, mainly associated with BPV-2, is related to bovine enzootic hematuria and urinary bladder carcinogenesis in adult animals grazing on pastures containing bracken fern (*Pteridium aquilinum*). This condition results from a synergistic interaction between the virus and carcinogenic compounds present in the plant. In buffaloes, this form has been reported in both Brazil and Italy (Maiolino et al., 2013; Roperto et al., 2013; Rocha et al., 2022).

In Brazil, cutaneous papillomatosis was first described in buffaloes in 1993 in females raised in the municipality of Registro, São Paulo State, through transmission electron microscopy and histopathological analyses (Cappellaro et al., 1993; Cappellaro, 1994). Subsequently, cases have been reported in several countries, including Italy, Iraq, Turkey, India, Mexico, and Brazil, and have been more commonly observed in adult animals, although occasional cases have been described in young individuals (Singh et al., 2010). Transmission occurs mainly from buffalo to buffalo, without the need for an intermediate bovine host, and is facilitated by injuries to the skin and mucous membranes caused by fences, water troughs, feeding troughs, or instruments used for animal restraint (Somvanshi, 2011). In addition to horizontal transmission, vertical transmission has also been reported (Russo et al., 2017). Different types of lesions may be observed, including papillomas, fibropapillomas, and fibromas (Jangir et al., 2017), as well as verrucous lesions affecting the mucosa of the rumen, oral cavity, and esophagus (Kumar et al., 2012), and even the amniotic membrane (Russo et al., 2020).

In general, papillomatosis is considered a self-limiting disease, with spontaneous regression of lesions occurring within approximately 12 months, depending on the host's cellular immune response. However, in some cases, malignant transformation may occur as a consequence of prolonged viral persistence within the host (Nasir & Campo, 2008).

Bovine papillomaviruses belong to the family *Papillomaviridae* and are non-enveloped, double-stranded DNA viruses with icosahedral symmetry and a diameter ranging from 45 to 55 nm (Freitas et al., 2011). Their capsid consists of 72 capsomeres, and the genome, approximately 8 kb in length, is organized into early (E1–E7) and late (L1 and L2) genes (Borzacchiello, 2008; Doorbar et al., 2012). To date, approximately 40 BPV types have been described infecting the skin, mucosal surfaces, and associated tissues (Elghmry et al., 2025). BPV types 1, 2, 5, and 6 are most frequently associated with fibropapilloma formation, whereas BPV-2 has been linked to urinary bladder tumors and amniotic lesions (Jangir et al., 2017; Maiolino et al., 2013; Rocha et al., 2022; Silva et al., 2026). The early genes E1 and E2 are involved in viral replication control and transcriptional regulation, while E5, E6, and E7 play key roles in cellular transformation and modulation of the host immune response (Borzacchiello et al., 2008; DiMaio & Petti, 2013). Among these proteins, E5 is considered the major BPV oncogene because of its ability to activate cellular proliferation pathways, particularly through interaction with growth factor receptors such as PDGFβR (Bocaneti et al., 2016). The late genes L1 and L2 encode the structural proteins of the viral capsid, with L1 being highly conserved among members of the *Papillomaviridae* family (Doorbar et al., 2012).

Papillomatosis is considered an emerging disease and causes substantial economic losses in intensive and semi-intensive dairy buffalo production systems. These losses are mainly associated with difficulties during milking, the presence of cutaneous lesions and bleeding, and an increased predisposition to mastitis. In meat production systems, the disease results in reduced weight gain, hide damage, and increased clinical complications, leading to higher veterinary care costs (Somvanshi, 2011; Rojas-Anaya et al., 2016). Furthermore, enzootic hematuria represents an important cause of economic losses in Brazil due to mortality and reduced productivity in affected animals (Rocha et al., 2022).

Given this scenario, the rapid diagnosis of papillomavirus infection in buffaloes is essential for disease control and for minimizing economic losses in production systems. The present study aimed to detect the presence of papillomavirus particles in cutaneous lesions of buffaloes using transmission electron microscopy techniques, providing ultrastructural characterization of the viral agent and contributing to the understanding of this disease in buffalo populations.

II. MATERIALS AND METHODS

2.1 Clinical Case

In 1993, samples of fibropapillomas located in the auricular region of five adult female Murrah buffaloes (*Bubalus bubalis*), aged between 18 and 20 months, from a farm located in the municipality of Registro, São Paulo State, Brazil, were sent to the Electron Microscopy Laboratory of the Biological Institute of São Paulo, São Paulo State, Brazil. The macroscopic appearance of all papillomas was that of spherical, grayish tumor masses measuring 0.5 to 2 cm, hairless, with a rough surface, elevations, and indentations.

2.2 Transmission Electron Microscopy

The samples were processed for transmission electron microscopy using negative staining, also known as rapid preparation, and resin embedding techniques.

2.2.1 Negative Staining Technique (Rapid Preparation)

During the negative staining procedure, fibropapilloma samples collected from the auricular region of buffaloes were first macerated and then suspended in 0.1 M phosphate buffer at pH 7.0, followed by transfer to sterile microtubes. Aliquots from the surface of these suspensions were deposited onto copper grids previously prepared with 0.5% collodion film in amyl acetate and reinforced with a delicate layer of carbon and were then allowed to adsorb briefly. Excess fluid was carefully removed with filter paper, and the grids were subsequently negatively stained with 2% ammonium molybdate at pH 5.0 (Brenner & Horne, 1959). At least three grids were prepared per sample, and multiple fields were examined to ensure representative sampling.

2.2.2 Resin Embedding Technique

The five fibropapilloma tissue fragments were initially fixed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer at pH 7.0, followed by post-fixation in 1% osmium tetroxide using the same buffer. Subsequently, the samples were dehydrated through a graded acetone series and embedded in Spurr resin (González-Santander, 1969; Luft, 1961). Ultrathin sections were produced with an LKB ultramicrotome and placed onto copper grids. Finally, the sections were stained with uranyl acetate and lead citrate (Watson, 1958; Reynolds, 1963). Multiple sections were examined per sample to ensure representative sampling of the tissue.

2.2.3 Electron Microscopy Examination

All grids were examined using a Philips EM208 transmission electron microscope at an accelerating voltage of 80 kV. Viral particles were identified based on morphological criteria including size, shape, capsomere arrangement, and presence of typical papillomavirus features. Particle diameters were measured using calibrated scale bars and image analysis software. A minimum of 50 particles per sample were measured to calculate mean particle sizes. Negative controls consisted of grids processed without sample material to monitor for contamination.

III. RESULTS

3.1 Clinical Case

Fibropapilloma samples obtained from buffaloes (Fig. 1, arrow) were prepared using the negative staining technique and subsequently analyzed by transmission electron microscopy with a Philips EM208 instrument. Papillomavirus particles were detected in all five samples examined (100% detection rate).

3.2 Transmission Electron Microscopy

3.2.1 Negative Staining (Rapid Preparation) Technique

In all five fibropapilloma samples examined by transmission electron microscopy using the negative staining technique (rapid preparation), papillomavirus particles, either isolated or grouped, were observed (Figs. 2-4). Their surfaces were composed of distinct subunits in symmetrical arrangements, with 15 to 17 capsomeres at the periphery, in which case they were interpreted

as intact or complete particles (Figs. 3, 4, large arrow). Often, however, the central portion was stained, and they were then considered empty particles (Figs. 3, 4, small arrow). The virions were non-enveloped and isometric, with an average diameter of 50.2 ± 3.8 nm (range 45–55 nm), consistent with previously reported measurements for bovine papillomaviruses (Freitas et al., 2011). No tubular particles were visualized.

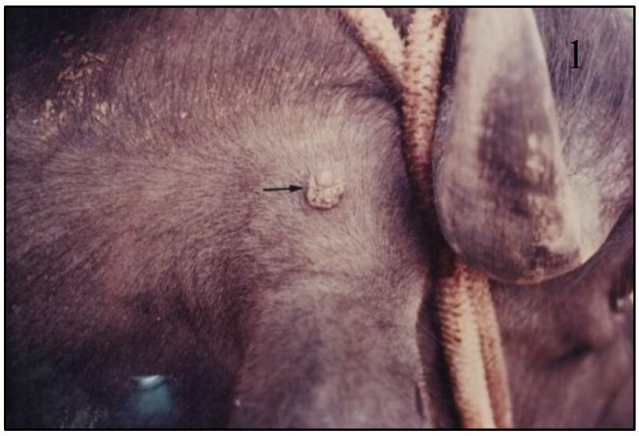

FIGURE 1: Photograph of a cauliflower-like papilloma (arrow), located at the base of a buffalo's ear.

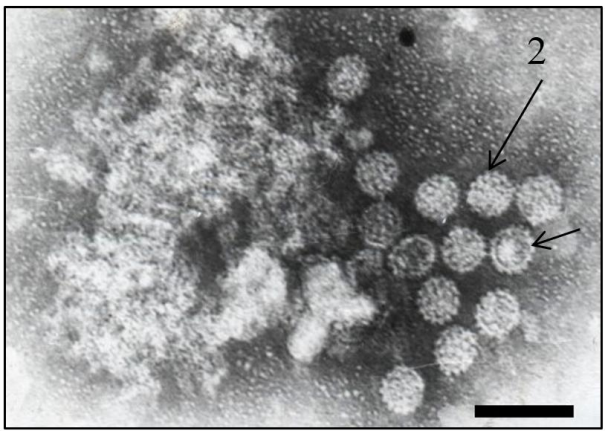

FIGURE 2: Negatively stained, non-enveloped, isometric papillomavirus particles, measuring an average of 50 nm in diameter (large arrow), showing individual capsomers (small arrow). Scale bar: 140 nm.

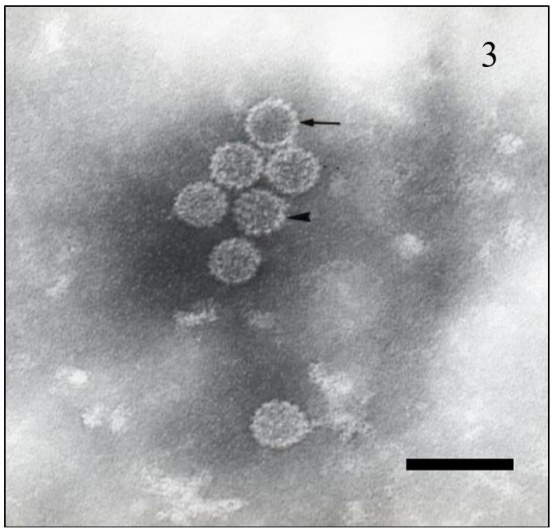

FIGURE 3: Negative staining of papillomavirus particles, showing "complete" particles (arrowhead) and "empty" particles (arrow). Scale bar: 120 nm.

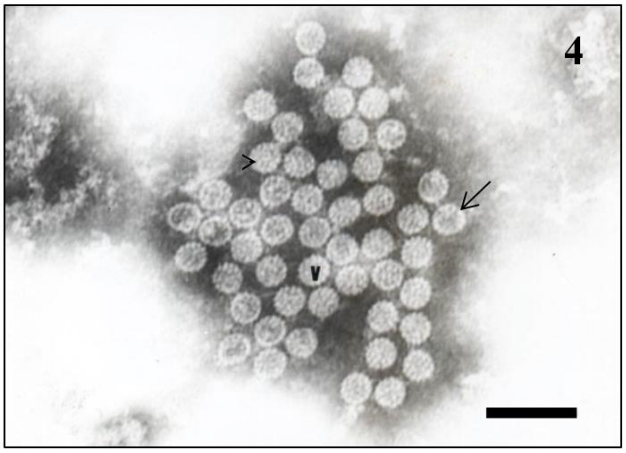

FIGURE 4: Negatively stained papillomavirus particles (v), non-enveloped and isometric, including "complete" (arrowhead) and "empty" (arrow) particles, measuring approximately 50 nm in diameter. Scale bar: 180 nm.

3.2.2 Resin Embedding Technique

In ultrathin sections of papilloma fragments, intracytoplasmic viral particles measuring approximately 30 nm in diameter (Fig. 5, large arrow) were observed in cells of the granular layer, associated with thickening of the plasma membrane, which exhibited a serrated appearance (Fig. 5, small arrow). In addition, intranuclear viral particles (Fig. 6, large arrow) and membrane-bound intracytoplasmic vacuoles (Fig. 5, arrowhead) were identified. In the reticular layer, a nucleus with an irregular contour, marginalized chromatin (Fig. 6, large arrow), and the presence of viral particles were observed (Fig. 6, small arrow).

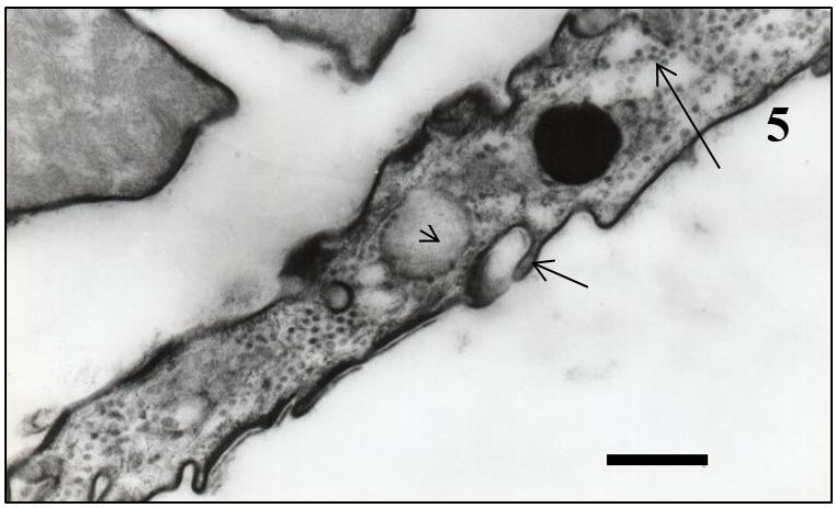

FIGURE 5: Ultrathin section of a papilloma showing a portion of a cell from the granular layer, with intracytoplasmic viral particles (large arrow) and thickening of the plasma membrane, which exhibits a serrated appearance (small arrow). Scale bar: 500 nm.

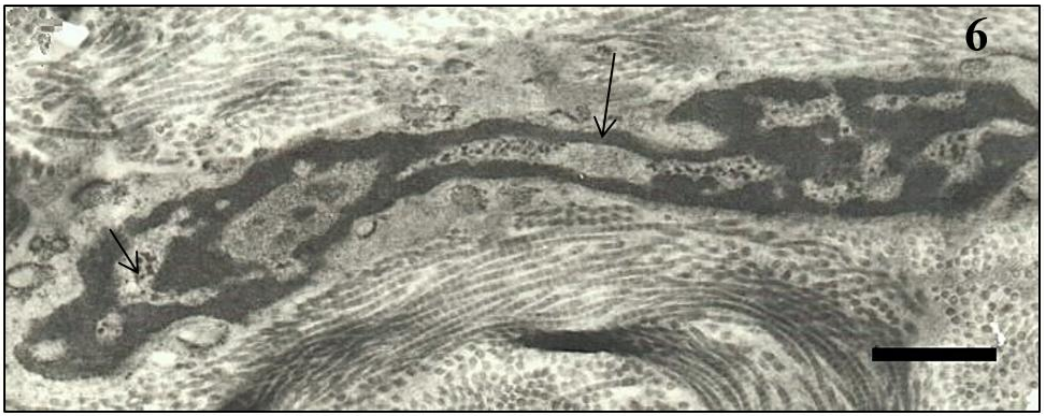

FIGURE 6: Ultrathin section of a papilloma showing, in the reticular layer, a nucleus with an irregular contour and marginalized chromatin (large arrow), as well as the presence of viral particles (small arrow). Scale bar: 620 nm.]

IV. DISCUSSION

In the present study, an ultrastructural investigation of cutaneous papillomatosis observed in buffaloes was conducted during an outbreak on a farm located in Registro, São Paulo State, Brazil.

The animals evaluated belonged to the Murrah breed, a finding that corroborates previous reports of papillomatosis in buffaloes described by Nooruddin et al. (1989), Prasad et al. (2009), and Singh et al. (2010). Furthermore, Raval et al. (2015) reported the occurrence of mammary fibroadenoma in a Mehsana buffalo raised in India, while Williams et al. (2011) described papillomatous lesions in African buffaloes (*Syncerus caffer*) in South Africa.

In this research, cutaneous papillomatosis was diagnosed in female buffaloes, a finding that is consistent with most previous reports involving this species (Singh et al., 2010; Williams et al., 2011; Raval et al., 2015; Russo et al., 2020; Rocha et al., 2022). Pangty et al. (2010) reported the occurrence of papillomas in 18 heifers, three adult female buffaloes, and two buffalo calves, whereas Maiolino et al. (2013) and Dileepkumar and Ansari (2012) described the disease in males. In an epidemiological study conducted by Nooruddin et al. (1989), involving 4,166 females and 284 males, the prevalence of papillomatosis was 0.36% in females and 0.7% in males. These findings indicate that the disease can affect animals of both sexes, with no apparent sex predisposition.

Regarding age, the buffaloes evaluated in the present study were between 18 and 20 months old. In previous investigations, the age of affected animals ranged from 6 to 18 months (Pangty et al., 2010), 1.5 years (Dileepkumar and Ansari, 2012),

approximately 2 years (Williams et al., 2011), between 3 and 5 years (Maiolino et al., 2013), 10 years (Raval et al., 2015), and 12 years (Prasad et al., 2009). Nooruddin et al. (1989) reported prevalences of 0.4% in animals up to 1 year of age, 0.35% in those between 1 and 3 years, and 0.38% in animals older than 3 years, indicating that both young and adult animals are susceptible to infection. Similarly, Singh et al. (2010) observed a higher frequency of the disease in adult animals, whereas younger individuals were less frequently affected.

It is noteworthy that, in the present study, papillomas were observed exclusively in the periauricular region. In contrast, several anatomical locations have been reported in buffaloes by different authors, highlighting the wide distribution of papillomatous lesions. These include the vulvar and perivulvar regions, back, and gluteal areas (Silvestre et al., 2009); teats, head, face, neck, legs, back, and urinary bladder (Roperto et al., 2013); legs and abdomen (Singh et al., 2010); the right eye (Singh & Mouli, 1990); the udder (Singh et al., 1991; Joshi et al., 1994; Raval et al., 2015); the face, eyelids, ears, neck, body, and trunk (Leishangthem et al., 2008; Dileepkumar & Ansari, 2012); the inguinal region, udder, groin, and thigh (Williams et al., 2011); the amniotic membrane (Russo et al., 2020); the rumen, reticulum, oral cavity, and esophagus (Kumar et al., 2012); the forehead (Prasad et al., 2009); the back, ears, knees, legs, limbs, and tail (Jangir et al., 2017); the head, neck, and udder (Al-Redah et al., 2021); and the urinary bladder (Roperto et al., 2013; Maiolino et al., 2013; Rocha et al., 2022). These reports demonstrate that papillomatosis may affect not only cutaneous surfaces but also mucosal tissues and internal organs.

Regarding their gross appearance, the papillomas presented as circumscribed, pedunculated tumor masses with a cauliflower-like appearance, a grayish, rough, fleshy surface, and absence of hair, features similar to those described by other authors (Singh et al., 2010; Pangty et al., 2010; Williams et al., 2011; Al-Redah et al., 2021). In previous studies, these lesions have also been described as small or large spherical nodules (Kumar et al., 2010), dome-shaped verrucous lesions, small growths or protuberances (Jangir et al., 2017), papillomatous nodules (Russo et al., 2020), fibropapillomas (Singh & Mouli, 1990; Joshi et al., 1994), warts (Leishangthem et al., 2008), and fibroadenomas or neoplastic masses (Singh et al., 1991; Prasad et al., 2009; Raval et al., 2015).

The tumor masses observed in the buffaloes examined in the present study ranged from 0.5 to 2 cm in diameter. In contrast, lesion sizes reported in previous studies have varied from 2.5 mm to 6 cm (Kumar et al., 2010; Pangty et al., 2010; Williams et al., 2011; Al-Redah et al., 2021).

Negative staining, also known as rapid preparation, was the method employed in the present study for the morphological characterization of viral particles, as it provides the most detailed visualization of these structures (Hazelton & Gelderblom, 2003; Goldsmith & Miller, 2009). Using this technique, isometric viral particles with an average diameter of 50.2 ± 3.8 nm were observed and classified as "complete" and "empty" particles. Similar findings have been reported in buffalo papillomas (Leishangthem et al., 2008; Silvestre et al., 2009; Singh et al., 2010), cattle (Catroxo et al., 2013; Módolo et al., 2017; Vrablikova et al., 2023), giraffes, and sable antelopes (Van Dyk et al., 2011). In contrast, Savini et al. (2020) were unable to visualize papillomavirus particles in ovine papilloma samples examined in their study. It is important to emphasize that successful application of the negative staining technique depends on the use of mechanically and thermally stable support films, reinforced with a thin carbon layer and applied to the metal grids prior to sample adsorption. This procedure provides greater stability to the specimen and facilitates the adequate visualization of viral particles (Gentile & Gelderblom, 2014).

In ultrathin sections of papilloma fragments, intracytoplasmic and intranuclear viral particles measuring approximately 30 nm in diameter were observed beginning in the stratum spinosum. These findings are consistent with those reported in buffaloes (Leishangthem et al., 2008; Singh et al., 2010; Williams et al., 2011; Roperto et al., 2013), cattle (Catroxo et al., 2013; Ozmen & Kale, 2023), dogs (Buff Jr. et al., 1988), and giraffes (Van Dyk et al., 2011). Pleomorphic nuclei with irregular contours and thickened, marginalized chromatin were also observed, similar to the ultrastructural alterations described by other authors (Leishangthem et al., 2008; Silvestre et al., 2009; Singh et al., 2010). The presence of membrane-bound intracytoplasmic vacuoles identified in the present study was likewise reported by Singh et al. (2010). However, unlike the findings of Singh et al. (2010) in buffaloes, we did not observe crystalline arrays of intranuclear viral particles. Such inclusions have been described in keratinocytes of papillomas in mice (Ingle et al., 2011).

The absence of molecular characterization (PCR, sequencing) to identify the specific BPV type represents a limitation of this study. The samples were collected in 1993, before molecular techniques were routinely available for viral typing in diagnostic laboratories. Based on the lesion morphology (fibropapillomas) and location (cutaneous), it is likely that the virus belongs to the genus *Deltapapillomavirus*, particularly BPV types 1 or 2, which are most commonly associated with fibropapillomas in cattle and buffaloes (Silvestre et al., 2009; Singh et al., 2010; Jangir et al., 2017). However, without molecular confirmation,

the specific BPV type cannot be definitively determined. This limitation should be addressed in future studies by applying PCR and sequencing to archived or new samples.

Buffalo papillomatosis is a highly contagious disease with a marked capacity for dissemination within herds and is considered endemic in buffaloes as well as in other livestock species. In addition to its sanitary and economic impacts, the disease has been regarded as a potential risk factor for both animals and humans (Jelinek & Tachezy, 2005). Several therapeutic approaches have been described for the treatment of papillomatosis, including autoimmunotherapy, surgical excision, cryotherapy, the use of antimony lithium thiomalate, and oral administration of *Thuja* extract in combination with the topical application of ointments containing this plant (Ghim et al., 2000; Dileepkumar & Ansari, 2012; Kavithaa et al., 2014; Lalrinkima et al., 2022).

The implementation of preventive sanitary measures is essential to minimize the spread of disease within production systems. The main control strategies include careful inspection of animals before their introduction into the herd, isolation of affected animals, periodic vaccination, proper cleaning and disinfection of milking equipment, and the use of disposable materials. Additionally, ectoparasite control, combined with the maintenance of high standards of hygiene and biosecurity, contributes significantly to herd health and the quality of animal production (Maeda et al., 2007).

Buffalo papillomatosis can result in significant economic losses, including reduced weight gain, decreased milk production, and alterations in meat and hide quality, thereby directly affecting the commercial value of the animals (Módolo et al., 2017). Therefore, early diagnosis is essential for the prompt implementation of control and preventive measures. In this context, the diagnostic techniques employed proved to be valuable tools for the rapid confirmation of papillomavirus infection, enabling high-resolution visualization of viral particles and characterization of the etiological agent during the outbreak affecting buffaloes.

V. CONCLUSION

This study confirms the occurrence of cutaneous papillomatosis in buffaloes in Brazil and demonstrates the utility of transmission electron microscopy for the rapid diagnosis and morphological characterization of bovine papillomavirus. The ultrastructural findings, including the visualization of non-enveloped, icosahedral viral particles with "full" and "empty" virions (45–55 nm), as well as intracytoplasmic and intranuclear viral particles (30 nm) and associated cellular alterations (serrated membranes, irregular nuclei with marginalized chromatin), provide detailed morphological evidence of papillomavirus infection in buffalo cutaneous fibropapillomas.

The results highlight the importance of electron microscopy as a diagnostic tool for emerging viral diseases in livestock. The high detection rate (100%) and the characteristic ultrastructural features confirm the presence of papillomavirus in the examined samples and contribute to the understanding of the pathogenesis of cutaneous papillomatosis in buffaloes.

Overall, this study provides valuable baseline information on the ultrastructural features of papillomavirus in buffaloes and emphasizes the need for integrated diagnostic approaches combining electron microscopy, histopathology, and molecular techniques for effective disease surveillance and control.

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DECLARATIONS

- **Ethics Approval**: Not applicable. This study was conducted on diagnostic samples submitted for analysis; no animal experimentation was performed.
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- **Author Contributions**: [To be specified by the authors]
- **Data Availability**: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

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