

Comparative Pathologic, Immunohistochemical, Ultrastructural and Molecular study of Bovine Papilloma Virus type 1 E5 Oncogene infection in Exotic and Indigenous cattle breeds

Olatunde B. Akanbi^{1,2} , Jens Peter Teifke³ , Adeyinka Jeremy Adedeji⁴ , Kati Franzke⁵ , Clement Adebajo Meseko⁴ , Oluwafemi B. Daodu^{5,6}  and Henry O. Jegede^{7*} 

¹Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ilorin, Ilorin, Nigeria

²Pathology unit, Laboratory medicine division, Veterinary Teaching Hospital, University of Ilorin, Ilorin, Nigeria

³Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institute, Greifswald - Insel Riems, Germany.

⁴Viral Research Division, National Veterinary Research Institute, P.M.B 1, Vom Plateau State, Nigeria.

⁵Institute for Infectiology, Friedrich-Loeffler-Institute, Greifswald - Insel Riems, Germany.

⁶Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Ilorin, Ilorin, Nigeria

⁷Zoo/Wildlife Unit, Veterinary Teaching Hospital, University of Ilorin, Ilorin, Nigeria.

*E-mail: drlanrejeg@gmail.com

ABSTRACT

Bovine papillomavirus (BPV) induces benign tumors of cutaneous or mucosa epithelia, called papillomas or warts in cattle and generally regress without eliciting any serious clinical problems in the host, but occasionally persist and provide the focus for malignant transformation to squamous cell carcinoma. This has a negative implication in beef and hide industry. There is paucity of information on the comparative pathology and molecular detection of BPVs in different breeds of nomadic cattle. Consequently, 340 nomadic cattle grazing in Plateau state Nigeria were screened clinically for papillomatosis followed by histopathology. Lesion consistent with papillomatosis were further investigated using immunohistochemistry (IHC), Polymerase chain reaction (PCR) and electron microscopy. Twenty cattle (0.06%) of Friesian, Zebu, Muturu and White Fulani breeds had cutaneous papilloma and skin lesions of the head, neck, shoulders, legs, dorsum, lower abdomen and scrotum. However, only 16 (0.05%) cattle skin biopsy samples were consistent with papillomatosis histopathologically revealing varying degrees of hyperplastic epidermis with acanthosis and orthokeratotic hyperkeratosis. Nuclei in the granular layer of the

epidermis were IHC positive for Bovine papillomavirus type I antigen while skin biopsy was BPV-1 E5 oncoprotein gene positive by PCR. Electron microscopy revealed ultrastructural changes consistent with Bovine papillomavirus infection. The distribution and severity of lesion varied in different breeds of nomadic cattle. The detection, diagnosis and characterization of papillomavirus in these cattle enabled the development of autogenous vaccine to immunized cattle. This study highlighted the comparative pathology and molecular characterization of BPVs in different breeds of nomadic cattle, which hitherto was lacking.

Keywords : BPV type I; papillomatosis; IHC; Ultrastructure; DNA E5 oncoprotein; Nigeria

INTRODUCTION

Cattle cutaneous papillomas are benign tumors caused by Bovine papillomaviruses (BPV) and generally regress without eliciting any serious clinical problems (Corteggio et al., 2013). In cattle, BPV types are classified into five genera with one unclassified strain: Deltapapillomavirus 4, Epsilonpapillomavirus 1, Xipapillomavirus 1, Xipapillomavirus 2, Dyoxipapillomavirus Dyokappapapillomavirus. They are further characterized as BPV-1 to BPV-23 (Bauermann et al., 2017; Borzacchiello et al., 2008; Daudt et al., 2018, Fagbohun et al., 2018). Of the several BPVs (BPV-1-23) characterized so far, BPV-1, BPV-2 and BPV-13, belonging to the delta (δ) papillomavirus genus 4, are commonly referred to as fibropapillomaviruses because they infect both the epithelium and the underlying dermis, giving rise to fibropapillomas (Jelinek et al., 2005)

Papillomas are usually a reaction of the skin to infection with papillomaviruses mainly affecting the shoulder, head and neck. Other areas often affected are teats and scrotum. The warts produced vary in shape and size from almost flat pea-sized lumps to large orange-sized balls on stalks and are seen as solid outgrowths of epidermis, sessile or pedunculated, with flat or cauliflower-like surfaces. They also appear as raised hairless lesions. Large warts bleed especially when rubbed, which can, in some instances, lead to secondary bacterial and fungal infection that may require treatment. BPV diagnosis usually includes a clinical examination, histopathology, immunohistochemistry and Polymerase chain reaction (PCR) has been used as a sensitive and specific method for the identification and genotyping of BPV (Betiol et al., 2012; Carvalho et al., 2012; Elzein et al., 1991; Leto et al., 2011; Nascimento et al., 2012)

Recent studies in Nigeria have shown that bovine papilloma is prevalent resulting in severe morbidity, lameness, loss of condition and reduced milk production (Fagbamila et al., 2012; Fagbohun et al., 2018; Jeremiah et al., 2016; Zwandor et al., 2008). Hides and skin devaluation and condemnations as a result of the disease constitute major loss to the leather industry. Nomadic cattle grazed in Vom (Longitude 9°43' 37"N and latitude 8°47' 21" E), Jos south local government area of Plateau state Nigeria were investigated for papillomatosis during a surveillance activity because information on the comparative pathology and molecular characterization of BPVs in different breeds of cattle is lacking. It is therefore of interest to identify and characterize the causative agent in these breeds.

MATERIALS AND METHODS

Study Area

Vom (Longitude 9.7269697 and latitude 8.7890627), a mountainous plain on an altitude of 1,217m above sea level with fertile land and abundant vegetation suitable for nomadic grazers is located in Jos south local government area of Plateau state, Nigeria. It enjoys a more temperate climate than the rest of Nigeria (average monthly temperatures of 21°C - 25°C) from mid-November to late January, night temperatures drop as low as 11°C resulting in chilly nights. Plateau State falls largely within the northern guinea

savannah zone which consists mainly of short trees, grasses and the Plateau type of mosaic vegetation. These conditions draw numerous nomads and their cattle herd to the area.

Animals

Three hundred and forty (340) nomadic cattle grazed in Vom were investigated for papillomatosis during a surveillance activity.

Clinical history and examination

The history of the animals was taken from the nomadic herders before clinical examination of the entire body including the skin, head, legs, udder, penis and oral mucosa was thoroughly performed.

Gross and Histopathologic examination

Cattle showing cutaneous papilloma and skin lesions were quarantined and biopsied. Histopathology involved skin biopsies of 0.5 x 0.5 cm in diameter (punched out of the skin) of affected Friesian, Zebu, Muturu and White Fulani breeds of cattle and were immersed in 10% phosphate buffered formalin fixative for 48 to 72 hours. The tissues were cut into blocks with a scalpel blade and placed in an automatic tissue processor for embedding. After a graduated dehydration in ethanol (70-100%), the tissues were cleared in xylene and finally embedded in paraffin. Sections of five micron (5µm) thickness were

prepared and thereafter, routinely stained with haematoxylin and eosin (H and E) stain as previously described (Akanbi et al., 2020). Stained tissue sections were histopathologically examined under a low and high powered field of Carl Zeiss® Axio Imager. A1 binocular microscope with an IC-3 mounted camera was used for photographing the microscopic lesions.

Immunohistochemistry

For immunohistochemistry, formalin fixed, paraffin-wax embedded (FFPE) sections (3µm) were dewaxed, rehydrated and were mounted on charged microscope slides (Menzel, Braunschweig, Germany). To detect bovine papillomavirus antigen, sections from each tissue block were evaluated by use of an avidin-biotin-peroxidase complex immunohistochemical staining method using a commercial rabbit polyclonal antibody against papilloma virus (PV) group-specific antigens (Dako Diagnostika, Hamburg, Germany) as earlier described (Borzacchiello et al., 2008; Teifke et al., 1998). In brief, sections were incubated with rabbit anti-bovine papillomavirus-1 antibody in a dilution of 1:3000 in Tris-buffered saline (TBS, 0.1 M Tris-base, 0.9% NaCl, pH 7.6). A biotinylated goat anti-rabbit IgG1 (Vector, Burlingame, CA; diluted 1:200 in TBS) was used as linker-antibody for the avidin-biotin-complex (ABC) method. As negative control, the pre-immunization serum of the same rabbit

was applied. Positive samples produce a bright red signal with an IHC kit (Vectastain Elite ABC Kit, Vector) and the substrate 3-amino-9-ethylcarbazole (DAKO AEC substrate- chromogen system; Dako, Carpinteria, CA, USA). The sections were counterstained with Mayer's hematoxylin and sealed with aqueous medium (Aquatex; Merck, Darmstadt, Germany). Sections of bovine papillomas and fibropapillomas, induced by Bovine Papillomavirus 1 (BPV 1, detected through PCR-analysis, DNA sequencing and ISH were used as positive control while normal epithelial tissue, present in most specimens or obtained from non-tumor-bearing animals, served as negative controls tissues of cattle were included for each immunohistochemistry procedure (Borzacchiello et al., 2008).

Ultrastructural technique

Electron microscopic examination was carried out using Tecnai® spirit transmission electron microscope as previously described (Akanbi et al., 2020). Briefly, small pieces of paraffin embedded skin biopsy tissue were chosen showing lesions after H and E stain in the light microscope. The paraffin was removed by heating the small pieces to 60 °C on filter paper for 15 minutes and subsequently incubated in xylene. Subsequently, tissue was rehydrated in ethanol and placed in 2.5% glutaraldehyde solution buffered in 0.1 M sodium cacodylate at pH 7.4 overnight. Osmium tetroxide was used

as second fixative and tissue was stained with uranyl acetate.

Finally, the samples were gradually dehydrated, transferred to a transitional solvent, propylene oxide, and infiltrated with epoxy resin (glycidyl ether). The polymerisation in gelatine capsules was carried out at 60 °C for three days (Akanbi et al., 2020). The ultrathin sections were placed on 300 mesh nickel grids and stained with uranyl acetate and lead citrate. For analyses a Tecnai G2 Spirit (FEI, Netherlands) transmission electron microscope was used at an acceleration voltage of 80 kV.

DNA Extraction and Purification from FFPE

Thick sections (5-10 µm) of BPV-1 positive FFPE cattle tissues were cut and DNA was extracted and purified using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). Briefly, 25mg of FFPE tissue samples from representatives of tissues from the different breeds of cattle were placed in 2ml Eppendorf tubes and 1.2ml xylene was added. Following full speed centrifugation at 25°C for 5 minutes, 1ml of 100% ethanol was added to the pellet to remove residual xylene. Following an additional centrifugation step, residual ethanol was removed by incubation at room temperature for 10 minutes after the main part of the supernatant was pipetted. The tissue pellet was resuspended in 180µl kit buffer ATL and was treated with 20µL

proteinase K. The purified DNA was resuspended in 100 µl of Kit Buffer AE.

Polymerase Chain Reaction Amplification of Viral DNA

DNA extracts from the representative samples of the Friesian, Zebu and Muturu breeds were used. The PCR mixture of 25 µl consist of 0.6 U/25 µl Taq DNA polymerase (Promega Corp., USA), 5µl DNA extract, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% TritonX- 100, 200 mM of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate, 0.25 mM of both primer pairs, 3.5 mM MgCl₂, and RNase free water. The cycling conditions was 95°C for 5 min, 30 cycles of 95°C for 30 sec, 63 °C for 30 sec and 72 °C for 30 sec followed by a final extension step 72 °C for 10 min. This was carried out in PTC-200 PCR-thermocycler (MJ Research Inc., USA). the primers set used (forward Oligonucleotide E5 primer 5'-CAA AGG CAA GAC TTT CTG AAA CAT-3' and reverse Oligonucleotide E5 primer 5'-AGA CCT GTA CAG GAG CAC TCA A-3') were complementary to a common sequence of the E5L2 open reading frame (ORF) of type 1 BPV (Borzacchiello et al., 2008). Cloned genomic DNA of BPV type 1 (pBPV-1; provided by Jens P. Teifke Friedrich Loeffler Institute, Insel-Reims, Germany) and DNA extracted from a bovine fibropapilloma and an equine

sarcoid were used as positive controls. Previously reported primer set targeted for the E5 gene were also for PCR amplification of all samples (Teifke et al., 1994; Teifke et al., 1998). Amplification products were detected by agarose gel electrophoresis.

RESULTS

Clinical history and examination

Three hundred and forty (340) cattle examined in the nomadic herds included 4 (1.2%) Friesian bulls, 35 (10.3%) Zebu breed, 31 (9.1%) Muturu breed, 232 (68.2%) White fulani breed and 38 (11.2%) were mixed breed. Twenty (0.06%) cattle breeds showing cutaneous lesions on the head, neck, shoulders, legs, dorsum, lower abdomen and or scrotum were subsequently classified.



Figure 1. A. Skin and face; Zebu yearling: Cutaneous papillomas, severe, diffuse masses of cauliflower-like exophytic, raised papilloma on the face, base of ears, mandibular region, head, and neck. B. face and neck; Muturu yearling: papillomas, moderate, multifocal single to coalescing cauliflower-like exophytic, raised papilloma on the face and neck. C and D. Friesian, moderate, multifocal single to coalescing cauliflower-like exophytic, raised papilloma on the face and neck. Some were found on the posterior hind limbs and perineum and presents, as discrete, often multiple to diffuse sometimes solitary to coalescing irregularly raised, pendunculated cauliflower-like growths (warts).

Gross and Histopathology

Sixteen cattle (4 Friesian, 7 Zebu, 3 Muturu and 2 White Fulani) with lesions typical of papilloma were screened (Table 1). Several cutaneous wart-like lesion of various diameter occurring on the head, neck, shoulders, legs, dorsum, lower abdomen and scrotum were grossly examined.

Grossly, lesions vary in the different cattle breeds. The Zebus showed the most severe and widely distributed lesion while those of Muturu breed were scattered and dispersed. The tumor masses were distributed on the head, neck and face, ears, mandibular regions, hump, shoulder, dorsum and the lateral abdomen (Fig. 1a). Some were found on the posterior hind limbs and perineum and presents, as discrete, often multiple to diffuse sometimes solitary to coalescing irregularly raised, pendunculated cauliflower-like growths (warts). Occasionally are foci of round, smooth and raised cutaneous nodules up to 2cm in diameter.

The masses were diagnosed as cutaneous papillomas on Hematoxylin and Eosin sections. Under the microscope, several layers of compact and laminated (hyperkeratosis) basket weave (orthokeratotic) keratinization (Fig. 2a and e; black thick arrows) were observed in the stratum corneum of the epidermis in all affected breeds of cattle and with increased severity in the Zebu breed (Fig. 2a). There is an excessive

epidermal hyperplasia with formation of rete pegs (Fig. 2a; black thin arrows) especially in the stratum spinosum giving the epidermis an irregular undulating configuration and projection of dermal papillae and epidermis above the surface of the skin (papillomatosis) (Fig. 2a). A higher magnification of the epidermis of the Zebu breed (Fig 2b), reveals an increased thickness of the stratum spinosum (Acanthosis) due to hyperplasia and occasionally to hypertrophy of cells of the stratum spinosum (Fig. 2b; black star). In addition, there was cell swelling, keratinolysis, keratohyalin granule retention, chromatinolysis, nucleolar degeneration, and presence of intranuclear (virions) appearing near perinucleolar chromatin. Also, loss of cohesion (Acantholysis) between epidermal cells resulting in intraepidermal clefts and vesicles were observed in the Friesian breed (Fig. 2e and f; black thick arrows). The presence of two to three (2-3) variably sized deeply basophilic intranuclear inclusions were commonly observed in the spinous strata of the Zebu and Friesian breeds (Fig. 2c and d; black arrow heads). Ballooning degeneration of the stratum spinosum as a result of intracellular edema, which is seen here as swollen eosinophilic cytoplasm, enlarged nuclei, and a loss of cohesion resulting in acantholysis (Fig. 2c and d; black thick arrows) and sometimes vesicle formation. In addition, many

koilocytes were often present, arranged in small clusters. These cells were depicted by large, mildly eosinophilic and vacuolated cytoplasm, and some cells comprised small distorted nuclei with dense chromatin. Nuclei in the majority of keratinocytes were vesicular, with fine chromatin and readily apparent nucleoli and some intranuclear inclusion bodies.

Ballooning degeneration is a characteristic feature of viral infections. The stratum granulosum within epidermis and surrounding the infundibula of hair follicles were obvious with flattened cells and their shrunken nuclei with deeply basophilic keratohyaline granules of variable size and shape (Fig. 2 c, d, f; black thin arrows).

Table 1. Clinicopathological and immunohistochemical (IHC) findings in Friesian, Zebu, Muturu and white Fulani breeds of cattle

Breed	Herd Population	Age/years	Clinical signs	Gross pathology	Histopathology	IHC
Friesian	4	7-9 years old bulls	Lethargy 4/4	Severe cutaneous papillomas 4/7	Moderate hyperkeratosis, ballooning degeneration and acantholysis 4/4	++
Zebu	35	Calf/yearling/Adult	Nil	Severe cutaneous papillomas 7/7	Severe hyperkeratosis, ballooning degeneration and acantholysis 7/7	+++
Muturu	31	Calf/yearling/Adult	Nil	Mild cutaneous papillomas 3/3	Mild hyperkeratosis, ballooning degeneration and acantholysis 3/3	++
White Fulani	232	Calf/yearling/Adult	Nil	Nil	Nil	Nil
Mixed	38	Calf/yearling/Adult	NA	NA	NA	NA

Notes : NA : Not applicable

IHC :Immunohistochemistry

+ Mildly positive

++ Moderately positive

+++ Very strongly positive

Immunohistochemistry

Immunohistochemistry revealed moderately to strongly positive cells to BPV-1 monoclonal antibody, visible as multifocally round, intranuclear, large

brownish red staining up to 10um in diameter, mainly in the junction of the corneum-spinosum area (Fig. 2g; red thick arrows).

Ultrastructural Pathology

Ultrastructural examination revealed the presence of two to three large intranuclear inclusions (virions) in virions producing cells. These virion-producing cells were only present at the spinous-corneum junctions. In the

stratum spinosum (prickle cell layer), there were separation of intercellular desmosomes and bridges between cells (Fig. 2h; red thick arrows). Also, loss of cohesion (Acantholysis) between epidermal cells resulting in intraepidermal clefts were observed. This is as a result of the breakdown of desmosomes between keratinocytes.

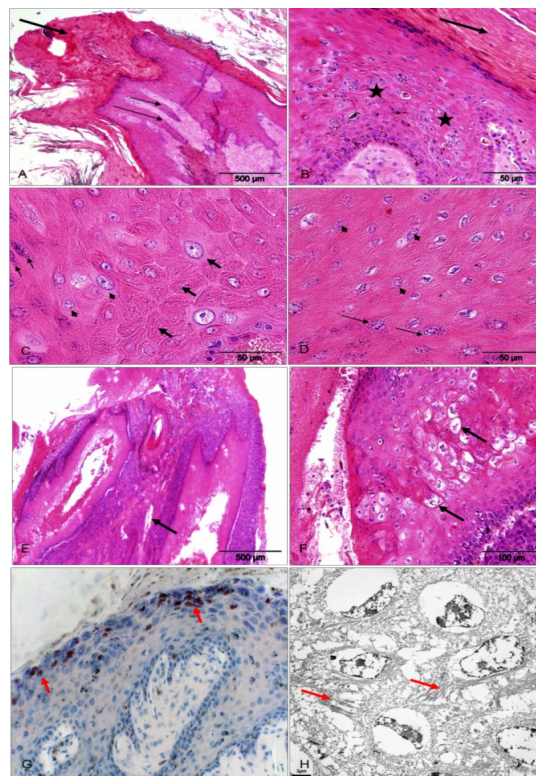


Figure 2. A. Zebu yearling, orthokeratotic hyperkeratosis (black thick arrows) and formation of rete pegs; B. excessive epidermal hyperplasia (black star). C. Ballooning degeneration of the stratum spinosum seen as swollen eosinophilic cytoplasm, enlarged nuclei, and a loss of cohesion resulting in acantholysis (black thick arrows) with presence of two to three (2-3) variably sized deeply basophilic intranuclear inclusions (black arrow heads). D. Deeply basophilic intranuclear inclusions (black arrow heads) and presence of deeply basophilic keratohyaline granules of variable size and shape. E. Friesian, acantholysis, intraepidermal clefts and vesicles (black thick arrows). F. Friesian, ballooning degeneration, acantholysis, intraepidermal clefts and vesicle

formation (black thick arrows).G. Intranuclear BPV-1 antigen immunopositivity of the granular and spinous cells of the epidermis to BPV-1 antibody. Immunohistochemistry (IHC). Bar= 200µm; H. Ultrastructural pathology revealed the presence of two to three large intranuclear inclusions (virions), separation of intercellular desmosomes and bridges between cells (fig. 2h; red thick arrows).

Polymerase Chain Reaction Amplification of Viral DNA E5 Oncoprotein

Following PCR amplification for BPV-1 E5, bands of 244 base pairs were detected in the DNA from three breeds

of cattle (Friesian bulls, Zebu and Muturu) except in white Fulani breed (Fig.3).

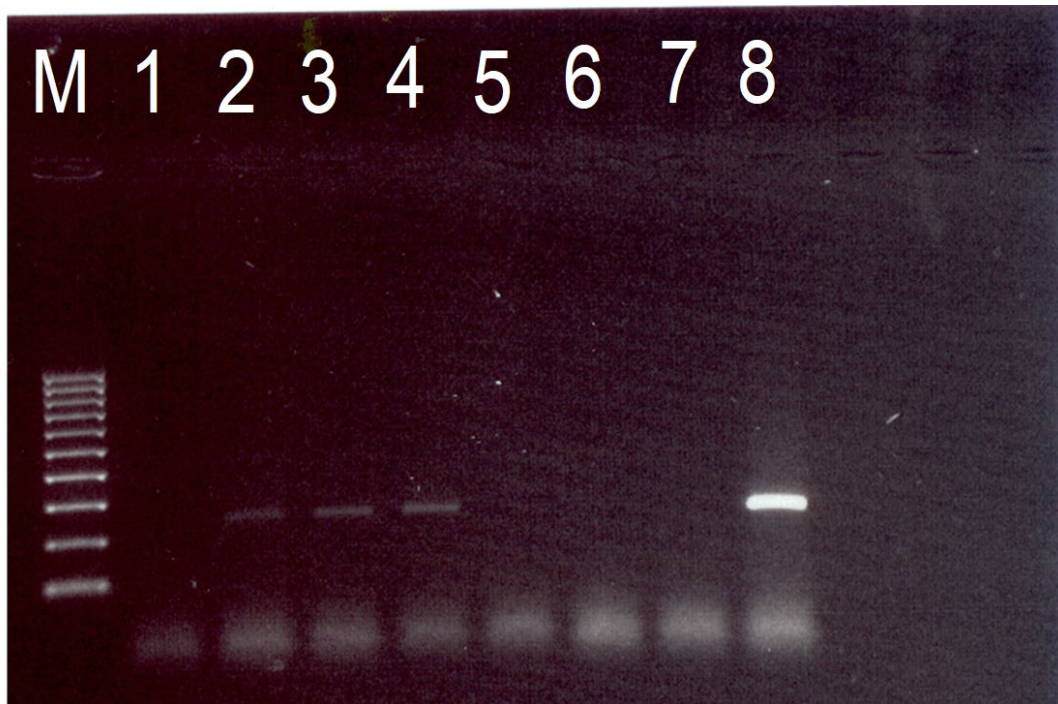


Figure 3. PCR Agarose gel electrophoresis: Lane M, Marker; Lane 1, nuclease free water (Negative control); Lane 2 Friesian; DNA sample ; Lane 3, (Zebu DNA sample; Lane 4, (MuturuDNA sample; Lane 5 (White fulaniDNA sample; Lane 6 (White fulani DNA sample; Lane 7 (White fulani DNA sample; Lane 8 known BPV-1 DNA(positive control).

DISCUSSION

Papillomatosis is a common but neglected disease with severe economic consequences in the beef, dairy and hide industries. In cattle, BPV types keeps increasing due to the discovery of new types (Bauermann et al., 2017; Borzacchiello et al., 2008; Da Silva et al., 2017; Daudt et al., 2018; Fagbohun et al., 2018; Lunardi et al., 2013a, Munday et al., 2015). Bovine papillomatosis has been described in the skin of cattle of all ages, and especially in young cows. This study documents the presence of cutaneous papillomas in Friesian, Zebu and Muturu breeds of nomadic cattle. While BPV-1, BPV-2, and BPV-13 induces fibropapilloma in diverse host species (Lunardi et al., 2013a, Lunardi et al., 2013b, Nasir & Campo, 2008; Nasir & Reid, 1999). This study found that different breeds in nomadic cattle are susceptible at varying degrees. In this herds the papilloma lesion is most severe and widely distributed on the skin in the Friesian and Zebu breeds, while the Muturu breed is infected with mild distribution and severity on the face of the animals affected. Although, the White Fulani cattle breed outnumbered the other breeds in this study, none showed any clinicopathological lesion. However, BPV-1 infection of the positive cattle breeds is evident in the histologic pattern, immunohistochemistry

demonstrating BPV-1 protein, PCR targeted at viral oncoprotein E5 gene and electron microscopy showing the intranuclear inclusions and virion. In all the samples, immunohistochemical detection of BPV-1 antigen was mainly in the nuclei of the cells of the junction of the stratum corneum and stratum spinosum. This is supported by previous study during experimental inoculation of papillomaviruses (PVs) into the skin which resulted in a biphasic response in basal keratinocytes (Cheville, 2014). This brought about keratinocyte hyperplasia and the resulting virus production, developed at the junction of the stratum corneum. This also explains why IHC detection was mainly at this junction (fig. 2g).

Cutaneous bovine papillomatosis is prevalent in the Nigerian livestock industry, and spread rapidly within herd either on farms or in cattle markets. It is also commonly seen during antemortem inspection at abattoirs. It is important to control the spread of the virus because of hide condemnation and the attendance economic loss to the farmers due to low priced hide by the leather processing industry.

There are few reports documenting bovine papillomatosis in Nigeria in the last 40 years. Previous studies in Nigeria reported papilloma and fibropapilloma in the West Africa Dwarf goats, severe cutaneous bovine

papillomatosis and co-infection in cattle (Fagbamila et al., 2010; Uzoukwu, 1979; Zwandor et al., 2008). The use of PCR and sequencing techniques were reported in Nigeria (Fagbohun et al., 2018; Jeremiah et al., 2016; Meseko, 2010).

It has been proven that both BPV-1 and BPV-2 are the etiologic agents of cutaneous papillomas in cattle (Hatama et al., 2008; Jarrett, 1985; Jelinek et al., 2005; Valarcher et al., 2008). In Nigeria, papillomatosis is prevalent on both intensive and free-range cattle herds and it is poorly reported for lack of concerted epidemo-surveillance and diagnostic work up (Fagbohun et al., 2018). This study has been able to provide information on the comparative pathology and molecular detection of BPVs in different breeds of nomadic cattle, which hitherto was lacking. The finding is of importance because, nomadic cattle are oftentimes grazed close together in search of lush and vegetation which this Vom mountainous plains with its fertile land and abundant northern guinea savannah zone vegetation provides. This attracts numerous nomads and their herds to this area, hence the spread of bovine papillomatosis amongst the herds. The finding of this study was also important to the development of an autogenous vaccine which was used to vaccinate the herds.

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