Molecular Characterization and Phylogenetic Analysis of Orf Virus Isolated From Goats in Sokoto Metropolis, Nigeria

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Aim: Despite the endemic nature of contagious ecthyma in Nigeria, there is limited report on the molecular characterization of the isolates responsible for disease outbreaks. The aim of this study was to molecularly characterize ORFV isolated from clinical infections in goats in Sokoto metropolis.

Materials and Methods: Seronegative embryonated chicken eggs were used to isolate ORFV via the chorio allantoic membrane (CAM) route according to the established protocol. Viral DNA was extracted from infected CAM and the full coding region of B2L gene was amplified by PCR and subsequently sequenced by Sanger's method. The nucleotide sequence results were blasted for identification and phylogenetically analyzed using MEGA and Bioedit softwares.

Results and Discussion: The results showed that B2L gene sequences of the ORFV UDUS/01/19/More strain showed slight variability (96- 98.7%) with the reference sequences. Our isolate clustered within the same clade with Korean strain signifying a close genetic relationship. Unique amino acid substitutions were noted in our isolate when compared with other references. This is arguably the first genetic characterisation of B2L gene of ORFV circulating in Nigeria.

Conclusion: Our study has provided in sight into the genetic diversity of ORFV in the study area. This is crucial for the design of effective vaccines against the disease which are currently lacking in the country.

Keywords: ORFV, PCR, Sanger method, B2L gene, Phylogenetic analysis

Background

Contagious ecthyma (CE) is a highly contagious viral disease of small ruminants such as sheep and goats (1) that occasionally affects camels (2) and wild ruminants with huge economic impact on the livestock industry (2–6). Clinically, the disease is associated with skin lesions such as erythema, macules, papules, vesicles,

 doi:10.20944/preprints202009.0663.v1

pustules and crusts on the lips, tongue, teat, nose, hooves and other parts of the body (6) especially in young lambs and kids (3,7). The disease is generally self-limiting, however, secondary bacterial infection may complicate the situation, causing in-appetence, severe emaciation and death of the affected animals (1). Epidemiological evidence indicated morbidity of 60% but the mortality is usually low unless complicated by secondary bacterial infection (8) where it can reach up to 10% and 93% in kids and lambs respectively (9) and even 100% in adult goats (4). The disease is also of zoonotic significance, causing ulcerative lesions or nodules on the hands of high risk individuals such as veterinarians, butchers and other animal handlers (10). Except in immunocompromised patients, most human cases of contagious ecthyma are localised and heal spontaneously (11).

The aetiology of CE is Orf virus (ORFV), a member of the genus *Parapoxvirus* in the family *Poxviridae* (12). The genetic material of the virus is a linear double stranded DNA (13) of 134-139 kb in size (3,13). It exhibits high GC content of about 66% (14) and is generally organised into conserved central portion and variable terminal regions (15). The central portion has a number of genes including the B2L gene, that encodes the immunogenic major envelope protein p42K (3,4,16,17). This gene has been extensively used for

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61 molecular detection and diagnosis (18) as well as phylogenic
62 analyses of various ORFV isolates (3,13,19).

63 Laboratory diagnosis of CE can be achieved using electron
64 microscopy, histopathology and serological tests such as
65 Fluorescent Antibody Technique (IFAT), Virus Neutralization Test
66 (VNT), Agar Gel Immunodiffusion (AGID) and Enzyme Linked
67 Immunosorbent Assay (ELISA) (1,3). Nowadays, confirmation of
68 CE is achieved using polymerase chain reaction (PCR) which has
69 been shown to be highly specific and sensitive (1,13,16,19). Using
70 PCR, sequencing and phylogenetic analysis, genetic characteristic
71 and diversity of ORFV has been described in many countries
72 around the world including China (5,19,20), Taiwan (13), Malaysia
73 (7), India (12), Uruguay (3) and few African countries such as
74 Tanzania (21), Ethiopia (17,22), Egypt (23,24), Gabon (25) and
75 Sudan (26,27). To date, outbreaks of CE in Nigeria are largely
76 reported based on clinical manifestation of the disease and PCR to
77 confirm cases (1,2,4) but no literature on the molecular
78 characterization of the circulating ORFV isolates is currently
79 available in Nigeria as at the time of this study. Therefore, in the
80 present study we reported for the first time the isolation, molecular
81 detection and phylogenetic characterisation of ORFV obtained from
82 a flock of goats in Sokoto metropolis, North Western Nigeria.

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Materials and methods

Sample collection, transport and processing

Suspected outbreak of CE was reported in a goat farm located in More area, Sokoto metropolis (13.0059° N, 5.2476° E) in May, 2019. On visitation to the farm, a flock of 30 goats consisting of Red Sokoto Goat (RSG) and crosses of RSG with West African Dwarf (WAD) was observed (8 males, 22 females). Five of the males were less than a year, while 3 were 2-3 years of age. The females on the other hand consisted of 10 adults (2-4 years) and 12 kids (less than a year). Physical examination of the two affected goats (2 year old male RSG-WAD cross and 3 year old female RSG) revealed scab lesions on the ears, lips, and nose (Figure 1). Two samples (1 and 2) involving thick brown scabs were scrapped on clean paper from the two affected goats and immediately transferred in a sterile sample container containing phosphate buffered saline (PBS, pH 7.2-7.4), placed on ice and immediately transported to the Central veterinary Research Laboratory, Usmanu Danfodiyo University Sokoto for analysis. After sample collecton, the two animals were isolated from the rest of the flock and were treated with long acting oxytetracycline 20% at 1 mL/20kg body weight to prevent secondary bacterial infection and the scrapped lesions were scrubbed with povidine iodine and sprayed with gentian violet to facilitate wound healing.

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The samples were homogenized in phosphate buffered saline (PBS) using tissue homogeniser and centrifuged at 1000 x *g* for 15 minutes to harvest the supernatants for storage at -20°C until needed for further analysis.

Figure 1: Sample collection from goats with suspected ORFV infection. Sample 1 from male RSG-WAD cross (Plate A) and sample 2 from female RSG (Plate B)

Virus isolation

Nine to eleven days old specific antibody free chicken embryonated eggs (CEE) were obtained from the Vaccine Research Division of the National Veterinary Research Institute Vom, Nigeria. The eggs were candled to ensure their viability and were later inoculated with 500 µL each of the two prepared samples via the chorioallantoic membrane (CAM) route as described by (28), sealed and labelled appropriately. The eggs were incubated and observed daily for 5 to 7 days during which eggs with dead embryos were chilled at 4°C. At the end of the incubation period, those embryos still alive were placed at 4°C overnight. Subsequently, the CAM was harvested and observed for the development of pock lesions before being stored at -20°C until processed.

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DNA extraction

Infected CAM harvested from the eggs inoculated with the two samples were used for genomic DNA extraction using DNA Mini kit (QIAGEN). Briefly, about 250 mg of CAM was homogenised and placed in a 1.5 ml micro centrifuge tube. Lysis buffer and proteinase K were added followed by incubation at 56° C in a water bath until complete lysis of the tissues occurs. DNA was then extracted according to the manufacturer's instructions, eluted with 50 ul elution buffer and stored at -20° C.

Polymerase chain reaction

The ORFVB2LF1 5′-TCCCTGAAGCCCTATTATTTTTGTG-3′ and ORFVB2LR15′-GCTTGCGGGCGTTCGGACCTTC-3′ specific forward and reverse primers described by Hosamani *et al.* (29) were used to amplify the complete B2L gene of the ORFV with the aid of Toptaq PCR mastermix (Qiagen) according to the manufacturer's instructions. The mixture was briefly centrifuged and placed in the thermocycler. Amplification was carried out using initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. Final extension was performed at 72°C for 5 min. The amplified products were then analyzed by electrophoresis on a 1.5% agarose gel containing 0.5 ng /ml ethidium bromide in TAE buffer. The amplicons were viewed using a GelDoc imaging system (BioRad).

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147 **DNA sequencing and phylogeny and evolutionary analysis**

148 PCR positive samples were sent to Inqaba, South Africa for Sanger
149 sequencing. The sequencing company reported that only one of the
150 two samples passed quality control (QC) for downstream
151 sequencing. Since the two samples were obtained from the same
152 outbreak in the same flock, we asked the company to go ahead and
153 sequence the sample that passed QC as the isolates in the two
154 samples are highly likely to be the same. On receiving the result for
155 the single sample, the Sequence was trimmed and subjected to
156 BLAST similarity search using the BLASTN algorithm of the NCBI
157 database, to confirm the identity of the virus. Subsequently, the
158 obtained sequence was deposited in the GeneBank database with
159 accession no MT272780. Reference sequences were downloaded
160 and aligned with the sequence obtained in this study using
161 ClustalW in the MEGA7 software (30). Phylogenetic tree was
162 constructed using the Neighbour-joining method with 2000
163 bootstrap replicates using MEGA7. Evolutionary distances were
164 inferred based on Pair Wise Sequence Comparison between the
165 isolates obtained in this study and the reference sequences.

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Results

Virus isolation

Following the inoculation of the processed two scab materials into the specific antibody free CEE, pathologic changes in form of small grayish white foci (pock lesions) were observed on the harvested CAM membranes. These changes were not observed in the mock inoculated eggs. This signifies the successful presumptive isolation and identification of the virus.

PCR and Sequence Analysis

The PCR amplification of the CAM homogenate from the two samples yielded a product of the B2L gene fragment at the expected band size of 1137 bp when analyzed by gel electrophoresis (Figure 2). The PCR product of sample number 2 from the RSG (well 4) had higher band intensity compared to sample number 1 from the RSG-WAD cross (well 3). None of the negative control samples showed a positive amplification.

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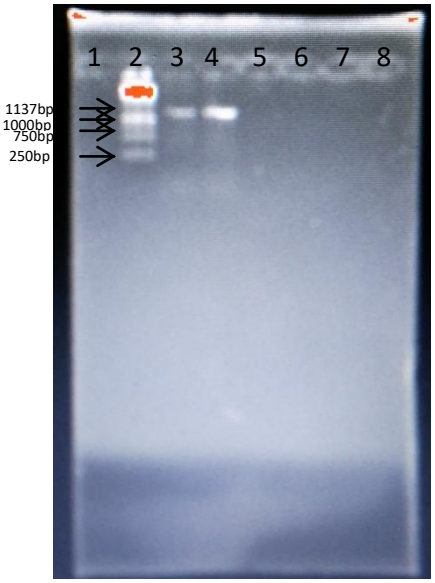

Figure 2: The PCR product showing the 1137bp fragments from sample 1 (well 3) and sample 2 (well 4, More strain) with the 1kb DNA ladder (well 2) and the negative control (well 5)

When the obtained PCR products were sent for sequencing, only sample 2 from the RSG (product in well 4) passed the quality assurance (QC) test necessary for a successful sequencing services possibly because of the high intensity of the band obtained by PCR amplification (Figure 2) and was therefore the only product sequenced in both directions using the B2L forward and reverse primers. Subsequently, the sequence obtained was subjected to BLAST search in the NCBI database and the identity was confirmed as ORFV which was named More strain. The sequence was aligned with downloaded ORFV reference sequences at nucleotide (Figure 3) and amino acid levels (Figure 4) to observe for similarities and differences in the nucleotide and amino acid sequences.

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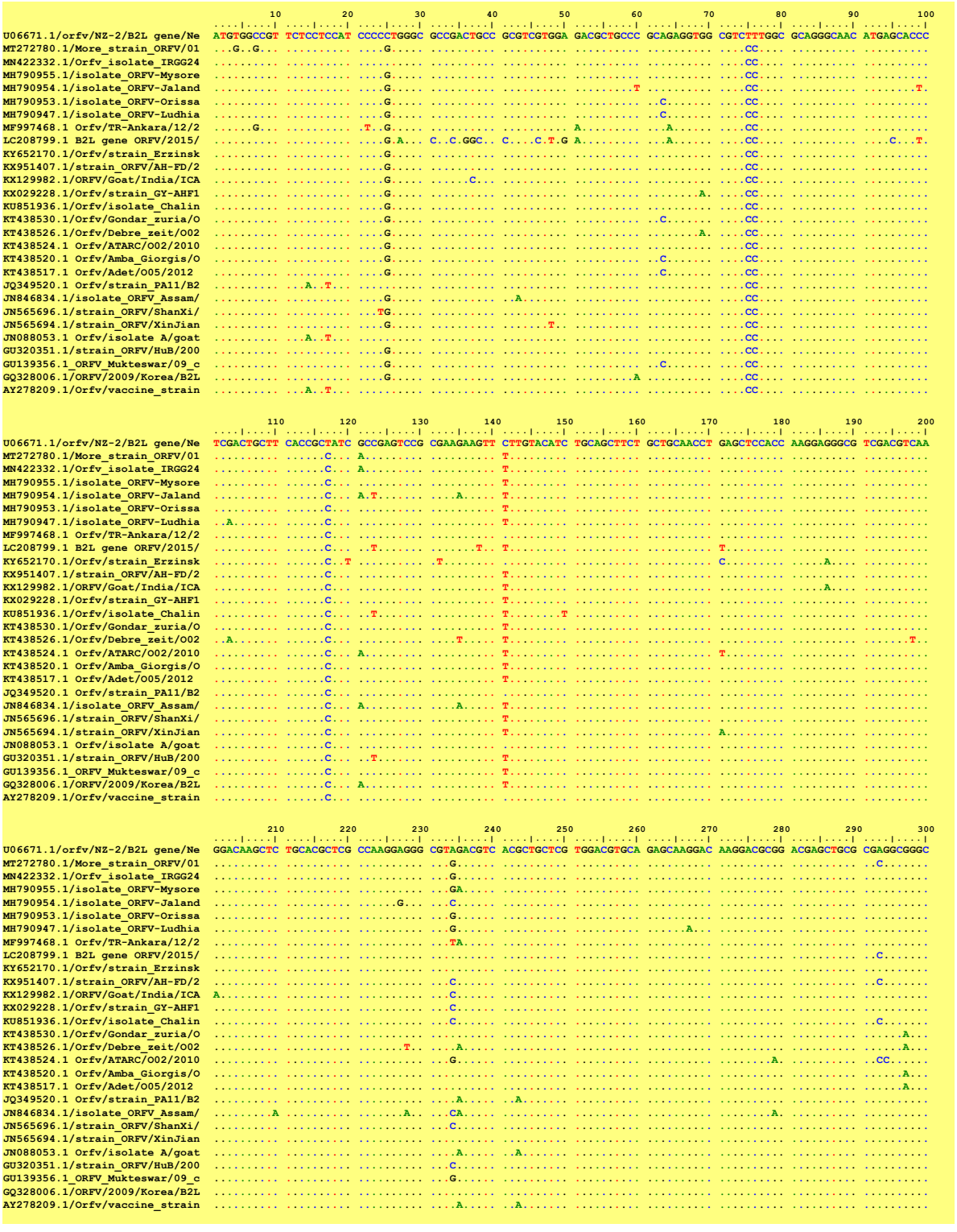

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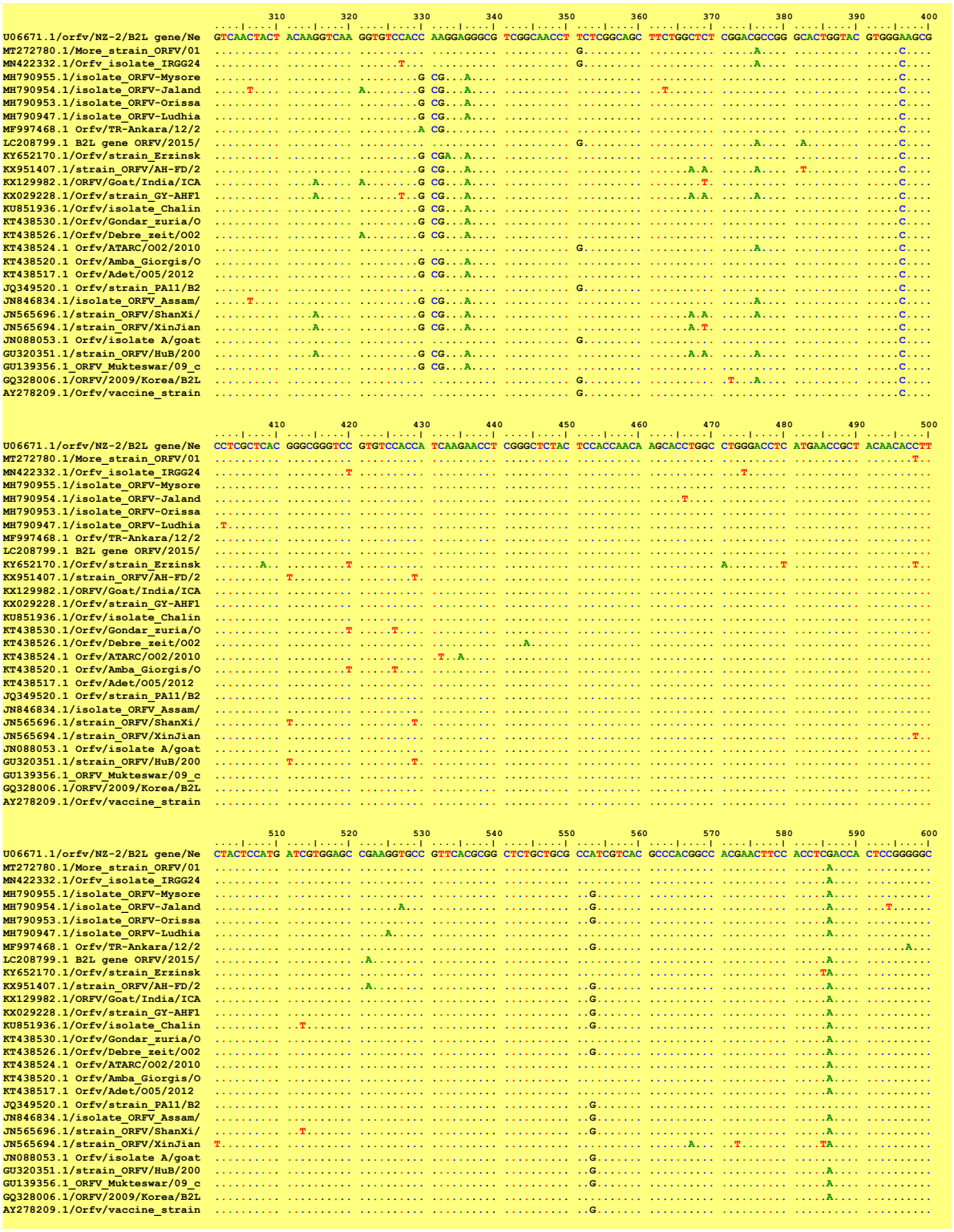
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LC208799.1 B2L gene ORFV/2015/
KY652170.1/Orfv/strain_Erzinsk
KX951407.1/strain_ORFV/AH-FD/2
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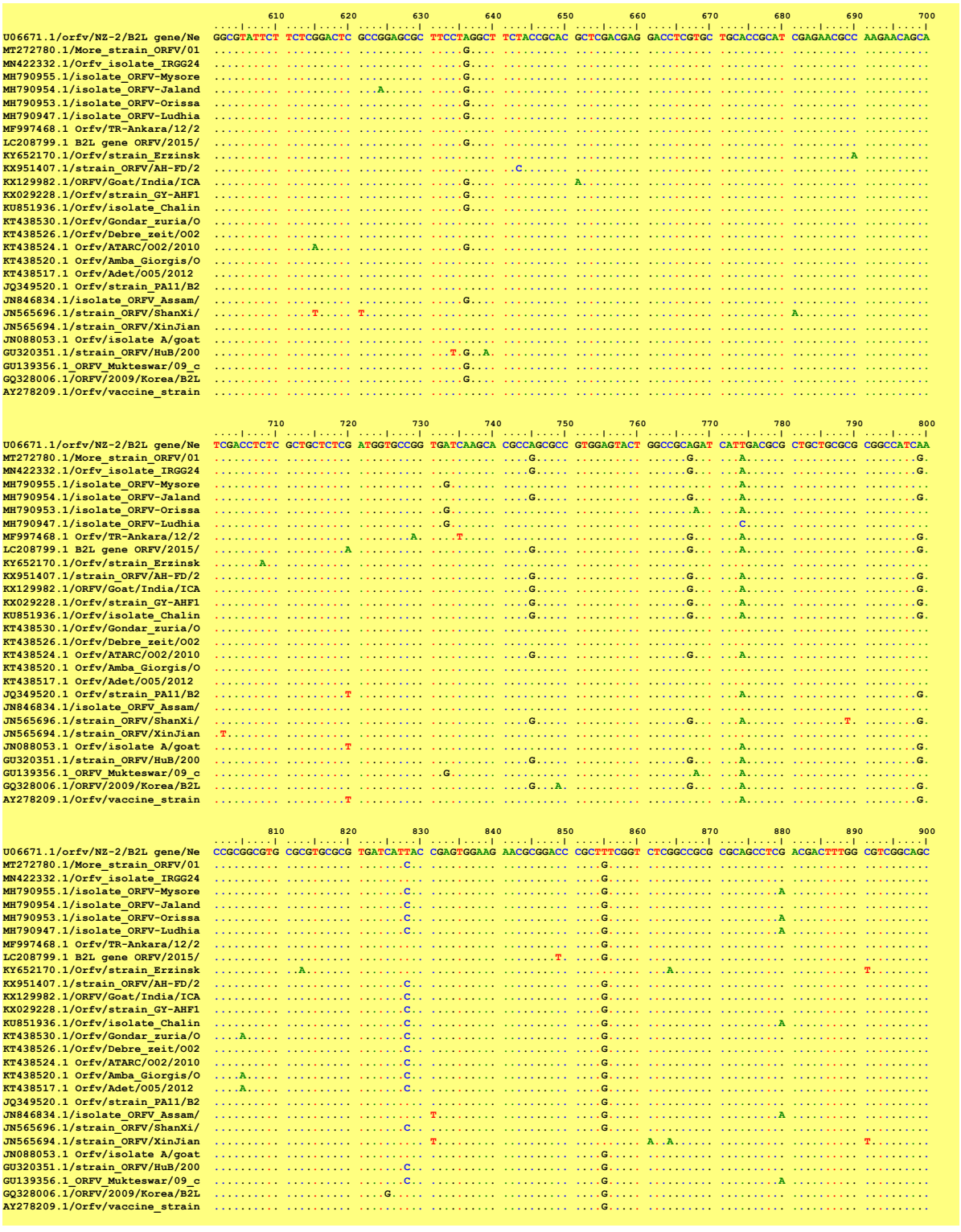
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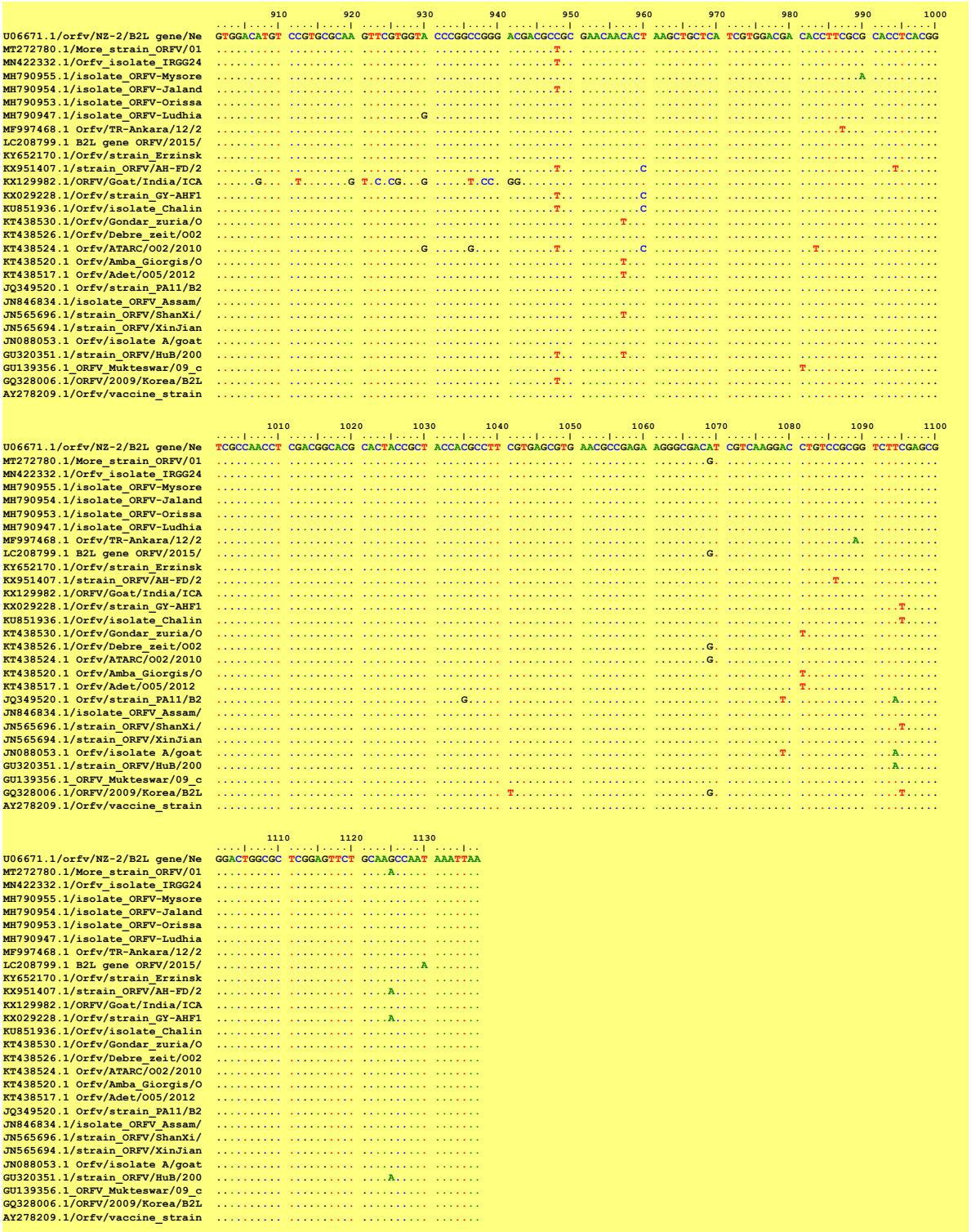

Figure 3: Nucleotide sequence comparison between More strain and reference sequences downloaded from the GeneBank, NCBI. The strain ORFV/NZ-2 from New Zealand (accession number U06671.1) was used as the guide sequence. Areas of similarity with guide sequence were represented as dots (….), while areas of differences were represented by a letter denoting the nucleotide

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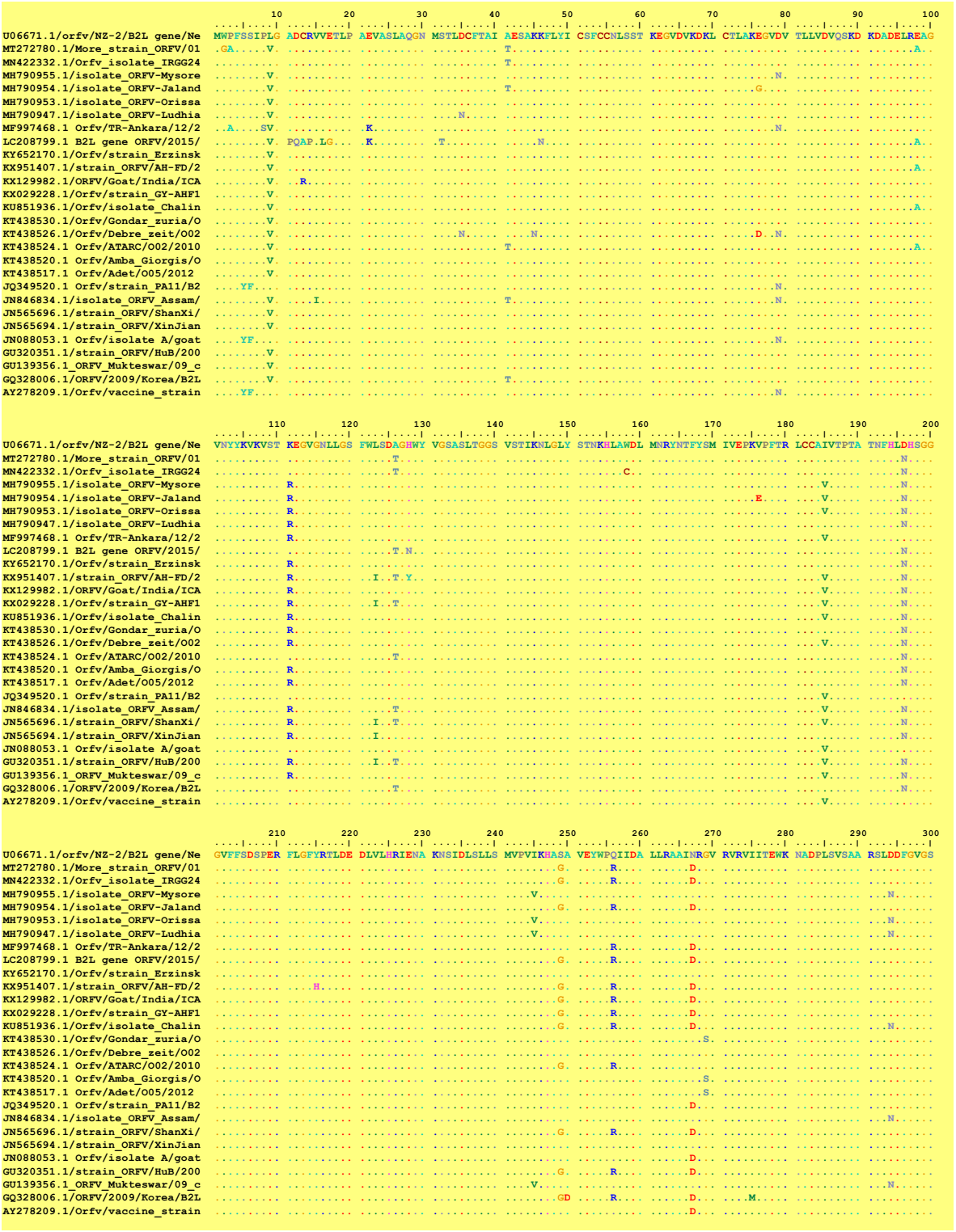

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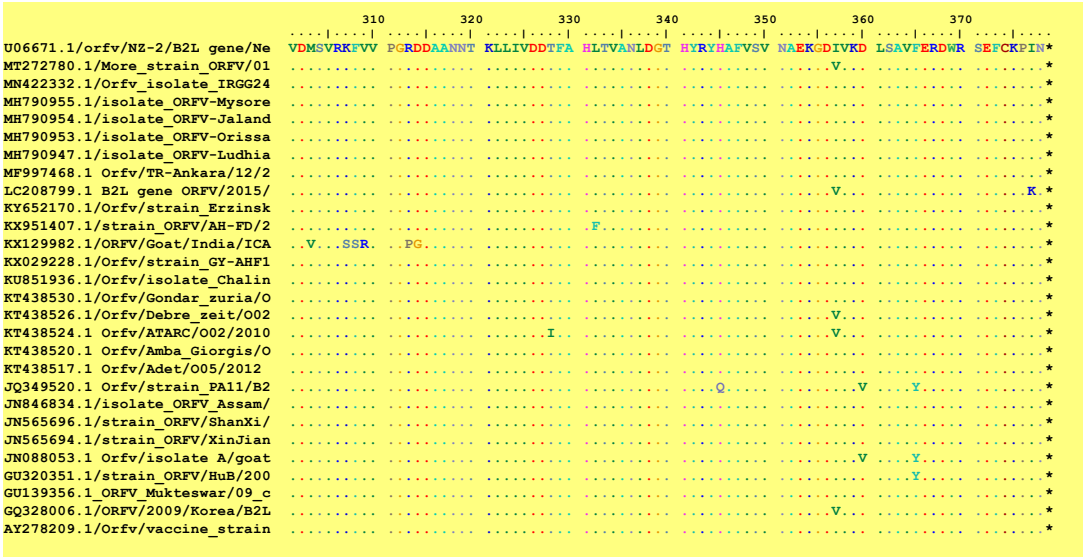

Figure 4: Amino acid sequence comparison between More strain and reference sequences downloaded from the GeneBank, NCBI. The strain ORFV/NZ-2 from New Zealand (accession number U06671.1) was used as the guide sequence. Areas of similarity with guide sequence were represented as dots (….), while areas of differences were represented by a letter denoting the amino acid

Comparison of the obtained sequence with reference sequences from different countries revealed nucleotide similarities range of 96.7-99.0% among the sequences. Similarly, at the amino acid level, the percentage homologies ranged from 95.7-98.9% between our isolate and the reference sequences. At the amino acid level, several mutations were observed in the More strain compared to the New Zealand NZ-2 strain used as a reference guide. There was a W2G at position 2 that was unique to the isolate studied, P3A present only in Ankara strain (MF997468), L9V present in almost all the other reference sequences, A41T which More strain share with 5 other reference sequences (MN422332; MH790954; KT438524; JN846834 & CQ328006), E98A found in 4 other reference sequences (LC208799; KX951407; KU851936 &KT438524) and A126T found also in 9 other reference sequences (MN422332; LC208799; KX951407; KX029228; KT438524; JN846838; JN565696; GU320351 & GQ328006). Other substitution mutations found were at amino acid positions D196N

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present in More strain and all other reference sequences except few (MF997468; JQ349520; JN088053 and the vaccine strain AY278209), S249G, Q256R, N267D and finally I352V (Figure 4).

Phylogenetic tree analysis based on B2L gene showed that More strain was closely related to isolate from Ethiopia (KT438524) which explained the high degree of nucleotide and amino acid similarities of 99.0% and 98.9% respectively that exist between them. They form a distinct cluster together with the isolates from Iran (MN422332), Korea (GQ328006) and Zambia (LC208799) (Figure 5). The Iranian strain was the most recent common ancestor to the Korean, Zambian, Ethiopian and More strains. Other isolates from diverse geographical locations (Asia, Africa, Europe, America and New Zealand) formed separate clusters and subclusters indicating a wide range of genetic diversity that exist in Orf viruses.

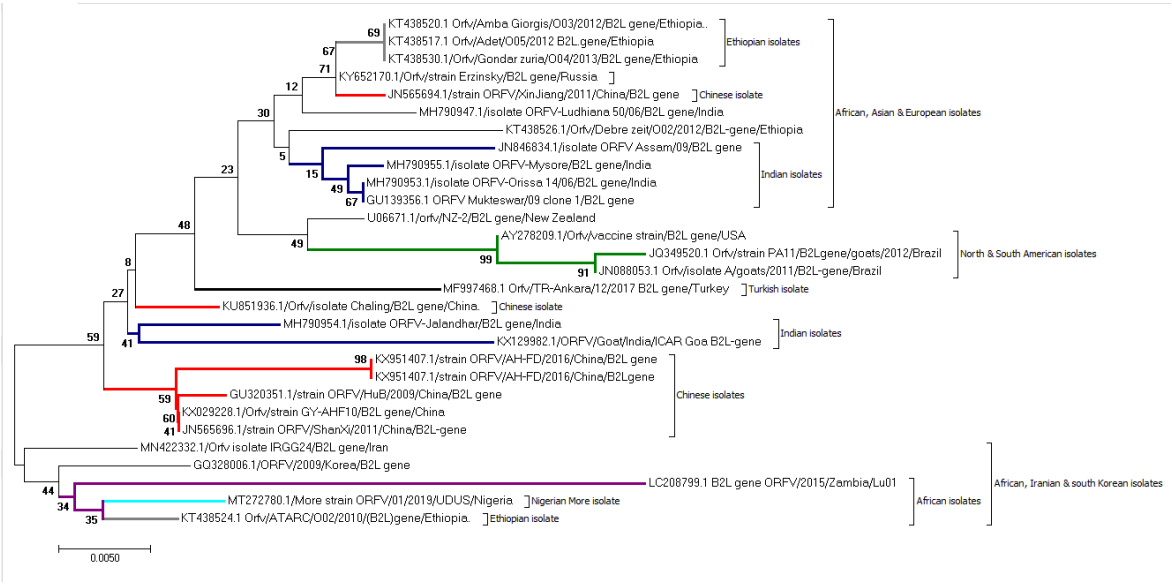

Figure 5: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 19 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 378 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

 doi:10.20944/preprints202009.0663.v1

Discussion

Orf or contagious ecthyma is endemic in Nigeria and causes huge economic losses in the National livestock industry (4). Effective control of the disease therefore demands early detection and identification of the causative agent. In Nigeria, outbreaks of CE are largely documented based on clinical signs which unfortunately may be confused with infection resulting from other viral disease agents such as sheep poxvirus, foot and mouth disease virus (FMD), bovine herpes virus type-2 and bluetongue virus. Thus, reliable estimate of the true prevalence of the disease in the country is currently lacking. In recent times, different techniques such as physical examination for clinical signs and PCR have been used to confirm contagious ecthyma outbreaks in Nigeria (1,2,4). Furthermore, previous study conducted in 2018 involving 3 flocks of goats in northern Nigeria reported a mortality rate of 100% (4) in two out of the 3 flocks investigated, which was higher than usually observed in CE outbreaks. This necessitated the need to molecularly characterise the ORFV circulating within the northern region of Nigeria to see how the Nigerian isolates may differ with other isolates globally. Moreover, to the best of our knowledge, no available literatures that molecularly describe the genetic characteristic of ORFV circulating in any part of Nigeria exist as at the time of this study. Consequently, the present study was

designed to isolate and genetically characterise the virus obtained from an outbreak involving a goat farm in Sokoto metropolis, North Western Nigeria.

Between May and September 2019, a number of suspected CE outbreaks have occurred in Sokoto State, Nigeria. One of those outbreaks involved a goat farm located in More village within Sokoto metropolis. Scabs were obtained from the two affected animals and prepared for inoculation into chicken embryonated eggs via the CAM route for virus isolation. Expectedly, the CAM developed characteristic pock lesions typical of Orf viruses, signifying virus replication. Although this method of virus isolation is simple and relatively rapid for Orf diagnosis, to the best of our knowledge, this is the first report of Orf virus isolation using specific antibody free chicken embryonated eggs in Nigeria.

Full length B2L gene fragments were obtained by PCR amplification and the full length sequence of one of the samples (sample 2) was obtained from Inqaba, South Africa. The other sample 1 was not sequenced because the PCR product failed the QC necessary for a successful sequencing. The sequence obtained confirmed the identity of the virus isolate as ORFV when blasted using the NCBI BLASTN tool. The virus when aligned with deposited ORFV sequences downloaded from NCBI database showed similarities with the reference sequences at both nucleotide

 doi:10.20944/preprints202009.0663.v1

and amino acid levels. A unique amino acid change observed only in the strain under study was the W2G seen at amino acid position 2. Another mutation observed was the P3A change at position 3 that was seen only in the Ankara strain (MF997468) from Turkey. However, the significance of these two substitution mutations as they affect the virulence and pathogenicity of the ORFV isolate under study need to be investigated.

Phylogenetic tree analysis based on the B2L gene showed two major branches on the tree. On the first branch, the IRGG24 (MN422332) isolate from Iran was placed at the root of this branch indicating that it is the most recent common ancestor from which other ORFV isolated from Korea (GQ328006), Zambia (LC208799), Ethiopia (KT438524) and the isolate under study originated. The position of the More strain (light green line) on this branch placed it more closely with the Ethiopian isolate (KT438524) which explained the high degree of nucleotide and amino acid similarities of 99.0% and 98.9% respectively that exist between the two isolates. The second branch on the tree had four major sub-branches where Chinese isolates (red line) diverged from the others, while the New Zealand (U06671), USA vaccine (AY278209) and two Brazilian strains (JQ349520 & JN088053) clustered together (green line) in a separate branch. Isolates from India (blue line) clustered together with one Ethiopian strain in a separate sub-branch. Finally, three

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Ethiopian isolates clustered with Erzinsky strain (KY652170) from Russia and XinJiang strain (JN565694) from China in a separate sub-branch. This analysis indicated that the Nigerian isolate investigated is genetically different with other ORFV isolated from different geographic regions of the world. However, the significance of this finding can only be fully understood when investigated further.

Conclusion

In summary, we have examined the genetic characteristics of ORFV causing disease outbreaks in Sokoto Metropolis, Nigeria and provided molecular signatures of the virus in the study area. We showed that the virus shared a close genetic relationship with an isolate from Ethiopia and harbours an amino acid change (W2G) that is unique to it and another mutation at P3A that is present in only in the Ankara strain (MF997468). This information is crucial to understand the molecular epidemiology of ORFV circulating in Nigeria, which is necessary for the design of effective CE vaccines which are currently lacking in the country.

Future Perspective

There is the need to undertake a state wide isolation and characterization study to fully understand the molecular epidemiology of the ORFV isolates circulating within the goat and sheep population in Nigeria. Whole genome sequencing can be done to add to the existing ORFV complete genome sequences since, currently very few are in the public database. The significance of the W2G and P3A mutations with respect to ORFV virulence modulation and pathogenicity needs to be investigated.

Financial and Competing Interests Disclosure

The authors have no competing interest or financial involvement with any entity or organization with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those already disclosed.

Acknowledgements

The authors acknowledged the contributions of the entire technical staff of the Central Veterinary Research Laboratory, Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto for their help in sample analysis.

Summary Points

- Scab lesions from goats suspected to be infected with ORFV based on clinical signs were obtained.
- Genomic DNA was extracted and amplified by polymerase chain reaction.
- The PCR products were analyzed by gel electrophoresis and sent for sequencing.
- The sequencing result was analyzed and aligned using ClustalW to determine its similarity with reference sequences downloaded from GeneBank.
- Deduced amino acid was determined and any change between the isolate and the reference sequences were noted.
- Phylogenetic tree was constructed to determine the taxonomic relationship between taxa.
- One unique amino acid change (W2G) and another (P3A) present only in a Turkish isolate were observed in the isolate under study.
- Phylogenetically, the isolate is more closely related to the Ethiopian isolate (KT438524) with which it cluster in the same clade different from other reference sequences.
- This is the first report on the molecular characterization of ORFV using full length B2L gene sequences.

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