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Dugbe Nairovirus S Segment: Correction of Published Sequence and Comparison of Five Isolates

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The sequence of the S (small) RNA segment of the ArD 44313 isolate of Dugbe nairovirus (DUG) has been redetermined, and a number of apparent errors in the previously reported sequence (V. K. Ward, A. C. Marriott, A. A. El-Ghorr, and P. A. Nuttall, 1990, *Virology* 175, 518–524) were revealed. Our results indicate that the S RNA is 1716 nucleotides (nt) in length and contains one large open reading frame spanning 1449 nt. This can encode a 483 amino acid polypeptide, M_r 53.9 kDa, corresponding to the viral nucleocapsid protein N. The DUG N protein is thus similar in length to the N proteins of Hazara (HAZ) and Crimean–Congo haemorrhagic fever (CCHF) nairoviruses, which are 485 and 482 amino acids in length, respectively. S segment RNA sequences were also determined for DUG isolates IbAr 1792, IbH 11480, ArD 16095, and KT 281/75; only the KT 281/75 sequence differed markedly from that of ArD 44313. Phylogenetic trees were constructed for these nairovirus S segment sequences.

Key Words: Dugbe nairovirus; family *Bunyaviridae*; S segment sequences; phylogeny.

INTRODUCTION

The *Bunyaviridae* family of viruses comprises over three hundred named isolates and is divided on the basis of serological and biochemical criteria into five genera: *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (Elliott *et al.,* 2000). Virus particles are spherical and enveloped and contain four structural polypeptides: two glycoproteins, G1 and G2, which are present on the surface of the virion, and two internal proteins, the nucleocapsid protein (N), and L, the virion-associated transcriptase (RNA polymerase). Viruses in some genera also encode nonstructural proteins. Viruses replicate in the cytoplasm of infected cells and bud primarily at membranes of the Golgi apparatus. They have negative-sense or ambisense RNA genomes comprising three segments, designated L (large), M (medium), and S (small). The infecting genome segments are replicated via positive-sense complementary RNAs (cRNAs) or are transcribed to give mRNAs. All members of the *Bunyaviridae* employ a "cap-snatching" mechanism similar to that of influenza virus in which mRNA synthesis is primed by cap-containing oligonucleotides generated by cleavage of host cell RNAs by a viral endonuclease (Elliott, 1990, 1996 and references therein).

The *Nairovirus* genus includes 34 named viruses and is divided into seven serogroups (Casals and Tignor, 1980; Clerx *et al.,* 1981; Calisher, 1996). The most serious human pathogen in the genus is Crimean–Congo haemorrhagic fever (CCHF) virus, which causes fatal haemorrhagic disease (Swanepoel *et al.,* 1987; Elliott, 1997). Recent outbreaks of this disease have occurred in regions as diverse as China, South Africa, Pakistan, and Russia (Fisher-Hoch *et al.,* 1995; Burt *et al.,* 1998). Another serious pathogen in the *Nairovirus* genus is Nairobi sheep disease virus, which is serologically related to Dugbe virus (DUG) and causes fever, haemorrhagic gastroenteritis, and abortion in sheep and goats in East Africa (Davies, 1997). In contrast, DUG causes only a mild febrile illness and thrombocytopaenia in man (Burt *et al.,* 1996). The principle arthropod vector of members of this genus is the tick, unlike most other members of the *Bunyaviridae*, which use mosquitoes or biting flies as vectors (Booth *et al.,* 1990; Calisher, 1996).

Dugbe virus is the best characterised member of the *Nairovirus* genus. Previous work has included the determination of the S (Ward *et al.,* 1990), M (Marriott *et al.,* 1992), and L (Marriott and Nuttall, 1996a) genome segments of isolate ArD 44313; thus far it is the only nairovirus whose genome has been completely sequenced. The S segment of Hazara virus (HAZ) isolate JC280 has been determined (Marriott and Nuttall, 1992), and the S and M segment sequences of several Crimean Congo haemorrhagic fever virus isolates are also known [Marriott and Nuttall, 1992; Marriott *et al.,* 1994; R. Lofts, L. Hodgson, J. F. and Smith, 1997, GenBank submission Em_vi:Chu88416]. Comparison of the published data for the DUG, HAZ, and CCHF S sequences indicated that the DUG sequence encodes a shorter N protein, 442 amino acids in length, than the related HAZ and CCHF viruses,

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which are 485 and 482 amino acids in length, respectively (Marriott and Nuttall, 1992). During the course of our recent work, we uncovered significant differences from the published DUG S sequence. We present here a corrected version of this sequence, together with a comparison of the S RNA sequences of five isolates of DUG and phylogenetic analysis of a representative number of nairovirus S segments.

RESULTS AND DISCUSSION

DUG S segment terminal sequences

During the course of our work we found differences at both the 3′ and the 5′ termini of the DUG S segment compared to published results. The terminal 3′ sequences of DUG ArD 44313 genomic RNA were originally determined by Clerx-van Haaster *et al.* (1982). These authors sequenced the 3′ terminal 15 nucleotide (nt) of seven different nairoviruses including DUG and found that the terminal 11 nts were generally 3′ AGAGUUUCU. . . . The DUG S 5′ genome terminus was determined by Ward *et al.* (1990) by a combination of Maxam–Gilbert sequencing and primer extension with dideoxynucleotides. Jin and Elliott (1993) extended this work by sequence determination of the 5′ termini of viral mRNAs (up to nt 300) produced in DUG IbAr 1792 infected cells.

The work by Jin and Elliott (1993) revealed a 2-nt (GC) insertion between nt 24 and 25 of the cRNA at the 3′ terminus of the DUG S genome RNA of IbAr 1792 compared to the published sequence of ArD 44313 (Ward *et al.,* 1990). We therefore analysed the ArD 44313 sequence in this region. The DgS1a–DgS5 PCR product, which encompasses nts 1–513 of the cRNA, was sequenced directly and found to contain the same GC insertion compared to the published sequence, as was seen for isolate IbAr 1792 (Fig. 1A). Moreover, this 2-nt insertion was also observed in the ArD 44313 clone PCRDUG8 kindly supplied by Professor Nuttall.

We then turned our attention to the 5′ end of the viral genome. We were unable to amplify viral cDNA at the 5′ terminus using primers based on the published sequence despite trying many different reaction conditions and using a number of different primer pairs. Alignment of the 5′-terminal regions of the DUG S, M, and L segments, together with those of the S segments of CCHF and HAZ (Fig. 1B), suggested that the DUG S sequence did not match particularly well with the other sequences. However, a new primer based on the predicted sequence for DUG S, DgS8b (5′ TCTCAAAGAGATCGTTACC), containing a 2-nt deletion (shown underlined in primer DgS8) compared to the original primer DgS8 (5′ TCTCAAAGAGAAATCGTTACCAC) was used to successfully amplify DUG S cDNA. A 2-nt insertion was also observed outside the primer region (between nts 21 and 22 in the published sequence, Fig. 1A). To sequence through the primer, we performed rapid amplification of cDNA ends (RACE) analysis on ArD 44313 RNA. First-strand cDNA synthesis was primed with DgS9; then an approximately 500-nt product was amplified using DgS4 and the dT primer supplied in the kit. Sequence analysis of the PCR product with an internal primer showed that position 17 of our sequence, 19 of the published sequence, is a G not an A, thus resembling HAZ and CCHF.

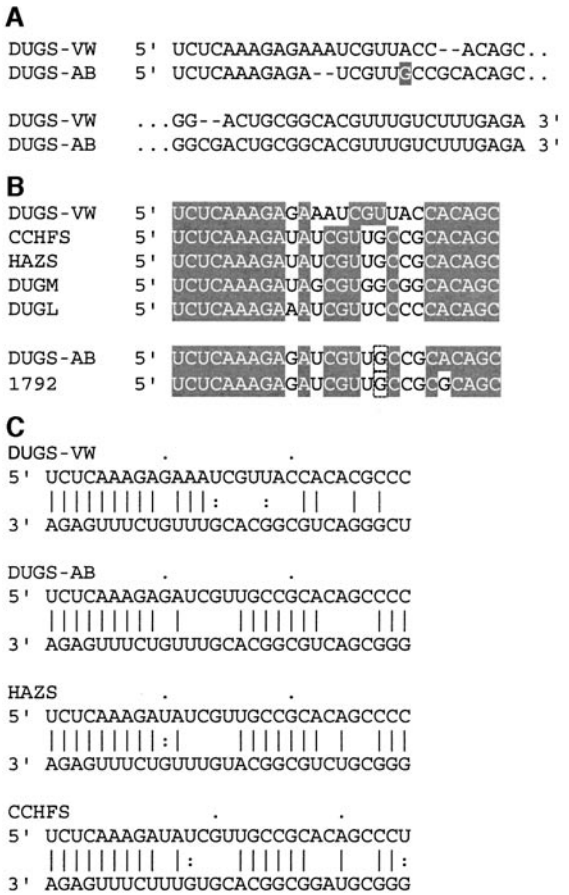

FIG. 1. Comparison of the Dugbe virus isolate ArD 44313 terminal sequences presented in this paper (DUGS-AB) with published data (DUGS-VW, Ward *et al.,* 1990), and the effect these differences have on the putative panhandles formed by annealing of both termini of the viral RNA. (A) Summary of the differences observed in the DUG S segment genome termini. (B) Alignment of the 5′ termini of all the DUG viral segments, together with the 5′ termini of other nairoviruses, used to predict the changes to the 5′ end of the Dugbe S segment. Shading indicates residues conserved between DUGS, CCHFS, HAZS, DUGM, and DUGL sequences. (C) Putative panhandles structures formed by the S genome RNAs of different nairoviruses.

A feature of the *Bunyaviridae* family is that their genomic RNAs are capable of forming panhandle structures because of the complementarity of their 5′- and 3′-terminal sequences. In Fig. 1C we show the predicted panhandle structures of the original and resequenced DUG S sequences, with those of HAZ and CCHF for comparison. With the newly determined DUG S-terminal sequence, the pattern of possible base pairs in the terminal 29 nt shows a greater resemblance to those of

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A
              residues 59-65    158-170          189-197      284-292
DUG-VW        KDSVFSN           VPGELLL.MSRML    GKKVLISRE    HLAKHVEL
DUG-AB        KDSIFSN           VPGEIAVDVKEML    GKKGPISRE    HLAQAVEL
CCHF-C        KDSIYAS           VPGEIVMSVKEML    P.RGPVSRE    HIAKAQEL
CCHF-AP       KDSIYAS           VPGEILVPVKEML    P.RGPVSRE    HIAKAQEL
HAZ           RDAIYSS           VPGTVINNIKEML    PKRGPVGRE    SYKTALAL
               :* *:*           *** :   :****     :**: **        *  *

              428                                                   483
DUG-VW        ASTIVQL..IQNPEIWL~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
DUG-AB        ASTIVQLYRIQKSGFDLESLEVVSTEHLLHQSFVGKRCPTQNAYKVRGNATV.NII
CCHF-C        AKTIVKLFEIQKTGFNIQDMDIVASEHLLHQSLVGKQSPFQNAYNVKGNATSANII
CCHF-AP       ARTIVKLFEIQKTGFDIKDMDIVASEHLLHQSLVGKQSPFQNAYNVKGNATSANII
HAZ           ACIISSLFEIQKAGYDIESMDIVASEHLLHQSLVGKRSPFQNAYLIKGNATNINII
              *  *  *: ***:*:::: :::*::*******:***:  * ****::****  ***

B
DUG-VW

          ACAATCGTGCAACTGATACAGAATCCAGAAATCTGGCTTTGATCTAGAAGCTTAGAAGTA
   1334   ------+---------+---------+---------+---------+---------+--- 1393
          TGTTAGCACGTTGACTATGTCTTAGGTCTTTAGACCGAAACTAGATCTTCGAATCTTCAT

a         T  I  V  Q  L  I  Q  N  P  E  I  W  L  *  S  R  S  L  E  V  -
b          Q  S  C  N  *  Y  R  I  Q  K  S  G  F  D  L  E  A  *  K  *  -
c           N  R  A  T  D  T  E  S  R  N  L  A  L  I  *  K  L  R  S  S -

DUG-AB

          ACAATCGTGCAACTGTACAGAATCCAGAAATCTGGCTTTGATCTAGAAAGCTTAGAAGTA
   1339   -+---------+---------+---------+---------+---------+-------- 1398
          TGTTAGCACGTTGACATGTCTTAGGTCTTTAGACCGAAACTAGATCtTTCGAATCTTCAT

a         T  I  V  Q  L  Y  R  I  Q  K  S  G  F  D  L  E  S  L  E  V  -
b          Q  S  C  N  C  T  E  S  R  N  L  A  L  I  *  K  A  *  K  *  -
c           N  R  A  T  V  Q  N  P  E  I  W  L  *  S  R  K  L  R  S  S -
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FIG. 2. (A) Predicted N protein sequence of DUG in regions where this differs from previously published results. The following deduced N protein sequences are aligned: previously and recently sequenced Dugbe isolate ArD 44313 (DUG-VW and DUG-AB, respectively), the CCHF isolates C68031 (from China, CCHF-C) and AP92 (originating from Greece, CCHF-AP), and Hazara isolate JC280 (HAZ). Differences in the DUG sequences are highlighted in bold type. Identical and conserved residues between the DUG-AB and other nairovirus sequences are indicated with asterisks and colons, respectively. (B) Nucleotide sequence of previously and recently sequenced Dugbe isolate ArD 44313 (DUG-VW and DUG-AB, respectively) with the corresponding amino acid translations in three frames. The DUG N ORF in DUG-AB and the corresponding amino acids in DUG-VW are underlined. Nucleotide differences between the two sequences leading to the frame shifts are shadowed.

the HAZS and CCHFS sequences, with more potential base pairing between nts 16 and 29.

Coding strategy of the DUG S segment

Sequencing of both clones and PCR products derived from the ArD44313 DUG S segment cDNA revealed a number of differences from published data. We observed a single open reading frame (ORF) between nts 52 and1500 of the S segment viral-complementary strand, which has the capacity to encode a protein of 483 amino acids. Previous work has demonstrated that this ORF encodes the N protein of the virus (Ward *et al.,* 1990). The effects that these nucleotide changes have on the predicted N ORF are summarised in Fig. 2A. The most significant of these occurs at the 3′ end of the N ORF and causes it to extend to nt 1495 instead of nt 1372 (equivalent to nts 1500 and 1379 in our sequence, respectively), thus increasing the predicted size of the N protein from 49.4 to 53.9 kDa (Fig. 2B). We have sequenced several PCR products, ArD 44313 pBluescript and pTM1 clones, which we have made in this region, and all sequencing results were consistent. In addition, we also sequenced the original pGM1636 clone provided by Professor P. Nuttall and this gave us the same result, thus indicating errors in the original sequence determination.

Further confirmation of these sequence findings was provided by *in vitro* transcription–translation reactions performed on two S segment clones. We had originally constructed a pTM1-DUGN expression clone by PCR amplification using primers DgS9 and DgS10 to express the ORF described by Ward *et al.* (1990), which extends from nt 50 to 1372 of the published sequence (nts 52–1379 in our sequence). Following our sequence results, we then constructed a similar but longer expression clone using primers DgS9 and DgS10a, which extends between nt 50 and 1495 (nts 52–1500 in our sequence). The primers DgS10 and DgS10a give rise to UGA and UAA stop codons, respectively, at the appropriate position on the complementary RNA strand. The results of this experiment are shown in Fig. 3. The cloned DgS9–

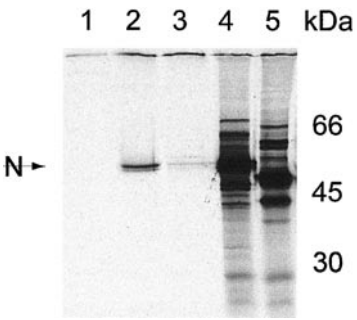

FIG. 3. Visualisation of the DUG N protein. The products from *in vivo* labelling of DUG-infected cells and *in vitro* transcription/translation of Dugbe virus N protein constructs were analysed on a 15% polyacrylamide gel. Lane 1: immunoprecipitation from mock-infected BHK cells using polyclonal rabbit anti-DUG antiserum; lanes 2 and 3, immunoprecipitation from DUG-infected BHK cells; lane 4: TNT reaction from the pTM1-N (nts 52–1500) clone; lane 5: TNT reaction from the pTM1-N (nt 52–1379) clone.

S10a product clearly expresses a larger protein, which moreover comigrates with a virus-specific protein in infected cells. This therefore confirms that the N ORF extends to nt 1500.

Close examination of Fig. 3 reveals the presence of two immunoprecipitated polypeptides from DUG-infected cells in the 53-kDa region. It is not yet clear whether these bands correspond to differently posttranslationally modified forms of the DUG N protein. Two virus-specific proteins of similar molecular weight were observed by Cash (1985), who identified the smaller of the two as the viral N protein on the basis of charge, whereas Ward *et al.* (1992) only observed a single N species.

Comparison of the S segments of five DUG isolates

The S genome segments of four additional isolates of DUG were sequenced completely from amplified cDNA and compared to the redetermined ArD 44313 sequence. The sequences of the Nigerian and Senegalese isolates (prototype ArD 44313, ArD 16095, IbAr 1792, and IbH 11480) were found to be very similar, with only 27 nt substitutions in the four S segments. Nucleotide identities between pairs of viruses were around 99%. The greatest sequence divergence was observed with the Kenyan KT 281/75 isolate, which showed only 91.2% nucleotide identity to the prototype ArD 44313 sequence.

The variation in the S segment sequence seen between different DUG isolates was noticeably less than that seen between CCHF isolates. For example, CCHF isolates 10200 (GenBank Accession No. U88410) and DAK 8194 (GenBank Accession No. U88411) isolated in Nigeria and Senegal, respectively (J. Smith, personal communication), the same countries of origin as the DUG isolates, show 82% identity. Two other African isolates, SPU-415 (GenBank Accession No. U88415) from South Africa and the 3010 Ugandan isolate show 84.6% identity to each other. Marriott and Nuttall (1996b) have also previously analysed partial CCHF sequences and noted the relatively large sequence variation between isolates.

All five isolates have an additional small ORF downstream, but slightly overlapping, the N ORF in the +1 frame. For the isolates ArD 44313, ArD 16095, IbAr 1792, and IbH 11480 this ORF extends from nt 1478 to 1627; for isolate KT 281/75 it is slightly shorter. There is as yet no evidence that this ORF is expressed.

One finding from the alignment made between the five sequenced DUG S RNA segments was that there was a 1- or 2-nt shift (dependant on the isolate) between the sequences encoding this possible ORF. This shift is illustrated in Fig. 4, which shows the entire cRNA sequence of each isolate from the N ORF UAA stop codon to the 3′ end of the cRNA. Each of the RNAs contains the same number of total nucleotides, but shifts are needed between the sequences to maintain the alignment. We believe these changes are real and not artifacts of the PCR process, since the sequence of each isolate was determined from the products of at least two independent PCR reactions. Variation in this region was also observed in the CCHF genome between isolates (see below).

Alignment of the S segments of all the CCHF S sequences present in GenBank (currently 15) shows that they vary immensely in the 3′ cRNA noncoding region. In particular, several contain deletions of varying length in this region, particularly the Ugandan 3010 isolate. Thus the CCHF-C68031, -AP92, and -3010 isolates have S segments of 1672, 1659, and 1640 nt, respectively, with the differences due to deletions in the NCR at the 3′ end of the cRNA, 5′ end of the genome. It thus suggests that there are fewer constraints on sequence divergence in this region of the nairovirus S segment than in the N ORF.

Phylogenetic analysis

The results given in Table 1 summarise the comparison of the DUG N protein sequence with those of CCHF and HAZ. The DUG N protein was 57.1% identical to the CCHF-C68031 N protein and 55.7% identical to the HAZ N. This is similar to the 60% identity seen between the CCHF-C68031 and HAZ N proteins.

To look more closely at the relationship between the different nairovirus sequences, we performed phylogenetic analysis on the five DUG S sequences presented in this paper, compared with HAZ S and three distinct CCHF sequences. Figure 5 shows the results of one such analysis; similar pictures were obtained using alternative alignment methods and also using protein instead of nucleic acid sequences. It suggests that the three nairovirus species, DUG, CCHF, and HAZ, form three distinct lineages, each of which is more or less equally distant from each of the other two viruses. The

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ArD 44313       TAAAAGCTGGCATTATGTGGCCTTCCCTAACATCTTAAACAAGTTGCACATCAGCGAAAG 60
ArD 16095       TAAAAGCTGGCATTGTGTGGCCTTCCCTAACATCTTAAACAAGTTGCACATCAGCGAAAG 60
IbAr 1792       TAAAAGCTGGCATTGTGTGGCCTTCCCTAACATCTTAAACAAGTTGCACATCAGCGAAAG 60
IbH 11480       TAAAAGCTGGCATTGTGTGGCCTTCCCTAACATCTTAAACAAGTTGCACACCAGCGAAAG 60
KT 281/75       TAAAAGCTGGCATTGTGTGGCCTGCCCTAACACTCTAGGCATGCTGCACATCAGCAAAAG 60
                ************** ******** ********   **  ** * ****** **** ****

ArD 44313       GCCACTAAAAGCTTTT-TTCTTACTTATCTTTCCATTTTTTTCTTCTTTATATATTAGTT 119
ArD 16095       GCCACTAAAAGCTTTT-TTCTTACTTATCTTTCCATTCTTTTCTTCTTTATATATTAGTT 119
IbAr 1792       GCCACTAAAAGCTTTT-TTCTTGCTTATCTTTCCATT-TTTCCTTCTTTATATATTAGTT 118
IbH 11480       GCCACTAAAAGCTTTT-TTCTTGCTTATCTTTCCATT-TTTCCTTCTTTATATATTAGTT 118
KT 281/75       GTCACAAAAAGTTTTCCTTCTT--TTATCTTCATATTCCCTTTTTCTTTATATATTAGTT 118
                * *** ***** ***  *****  *******   ***   *  *****************

ArD 44313       TTGCTTTTT-GACTAACATACTAACATAGTGTAAGTTGTAATCAATCTGCTAATTCTGCT 178
ArD 16095       TTGCTTTTT-GACTAACATACTAACATAGTATAAGTTGTAATCAATCTGCTAATTCTGCT 178
IbAr 1792       TTGCTTTTTTGACTAACATACTAACATAGTGTAAGTCGTAATCAATCTGCTAATTCTGCT 178
IbH 11480       TTGCTTTTTTGACTAACATACTAACATAGTATAAGTTGTAATCAATCTGCTAATTCTGCT 178
KT 281/75       TTGCTTTTTTAACTAACATACTAAAATAGTATAAGTTGTAATCAATTTGCTAATTCTGCT 178
                *********  ************* ***** ***** ********* *************

ArD 44313       TAACACAGGGGGGCTGTGCGGCAACGATCTCTTTGAGA 216
ArD 16095       TAACACAGGGGGGCTGTGCGGCAACGATCTCTTTGAGA 216
IbAr 1792       TAACACAGGGGGGCTGCGCGGCAACGATCTCTTTGAGA 216
IbH 11480       TAACACAGGGGGGCTGTGCGGCAACGATCTCTTTGAGA 216
KT 281/75       TAATACAGGGGGGCTGTGCGGCAACGATCTCTTTGAGA 216
                *** ************ ********************
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FIG. 4. The figure shows an alignment of the S segment cRNA sequences downstream from the N ORF for the five isolates of Dugbe virus described in this paper, starting from the UAA stop codon. Asterisks indicate nucleotides conserved between all five isolates, and gaps in the alignment are shown with a dash.

five DUG isolates are much more closely related to each other than are the CCHF isolates, and the DUG isolates are related according to their country of origin.

CONCLUSIONS

We have provided evidence for sequence errors in previously published data for the NCR of the DUG S sequence and also in the ORF encoding the viral N protein. This therefore increases the predicted size of the N protein from 49.4 to 53.9 kDa. We have compared the S segment sequences of five isolates of DUG and found slight differences between the isolates originating in Senegal and Nigeria. The Kenyan KT 281/75 was the most distinct isolate. Phylogenetic analysis suggests that the three nairovirus species sequenced to date, DUG, CCHF, and HAZ, are approximately equally distant from each other in evolutionary terms.

TABLE 1

Relationship between Nairovirus N Proteins

	DUG ArD 44313	CCHF AP92	CCHF C68031	HAZ JC280
DUG ArD 44313	—	66.2	66.0	65.2
CCHF AP92	57.3	—	95.0	69.7
CCHF C68031	57.1	91.7	—	69.9
HAZ JC 280	55.7	59.3	60.0	—

Note. Percentage amino acid similarities (top half of the table) and identities (lower half of the table) of selected nairovirus nucleocapsid proteins. Analyses were made using the University of Wisconsin GCG package BESTFIT, with default parameters (gap weight = 8, length weight = 2).

MATERIALS AND METHODS

Viruses and cells

The five DUG virus isolates used in this study included two from Nigeria, the prototype strain IbAr 1792 (David-West and Porterfield, 1974) and IbH 11480, two isolates from Senegal, ArD 44313 (Ward *et al.,* 1990) and ArD 16095 (Sweet and Coates, 1990), and the Kenyan KT 281/75 isolate (Cash, 1985). Mouse brain stocks of these isolates were a kind gift from Dr. E. Gould, Institute of Virology and Environmental Microbiology (IVEM), Oxford. Viruses were propagated in BHK cells at 31°C using an m.o.i. of 0.1 and then titrated by plaque assay on XTC-2 cells (Watret *et al.,* 1985).

RNA extraction, reverse transcription, and PCR

Supernatants from infected cells were harvested at 6–7 days postinfection, and viral RNA prepared with Trizol as described previously (Bridgen and Elliott, 1996). RNA was reverse transcribed with Superscript II (GIBCO-BRL) using primer DgS1 (5′ TCTCAAAGACAAACGTGC-CGCAG) or DgS9 (5′ GACAGGAAGACAGCATGGAGAAT-

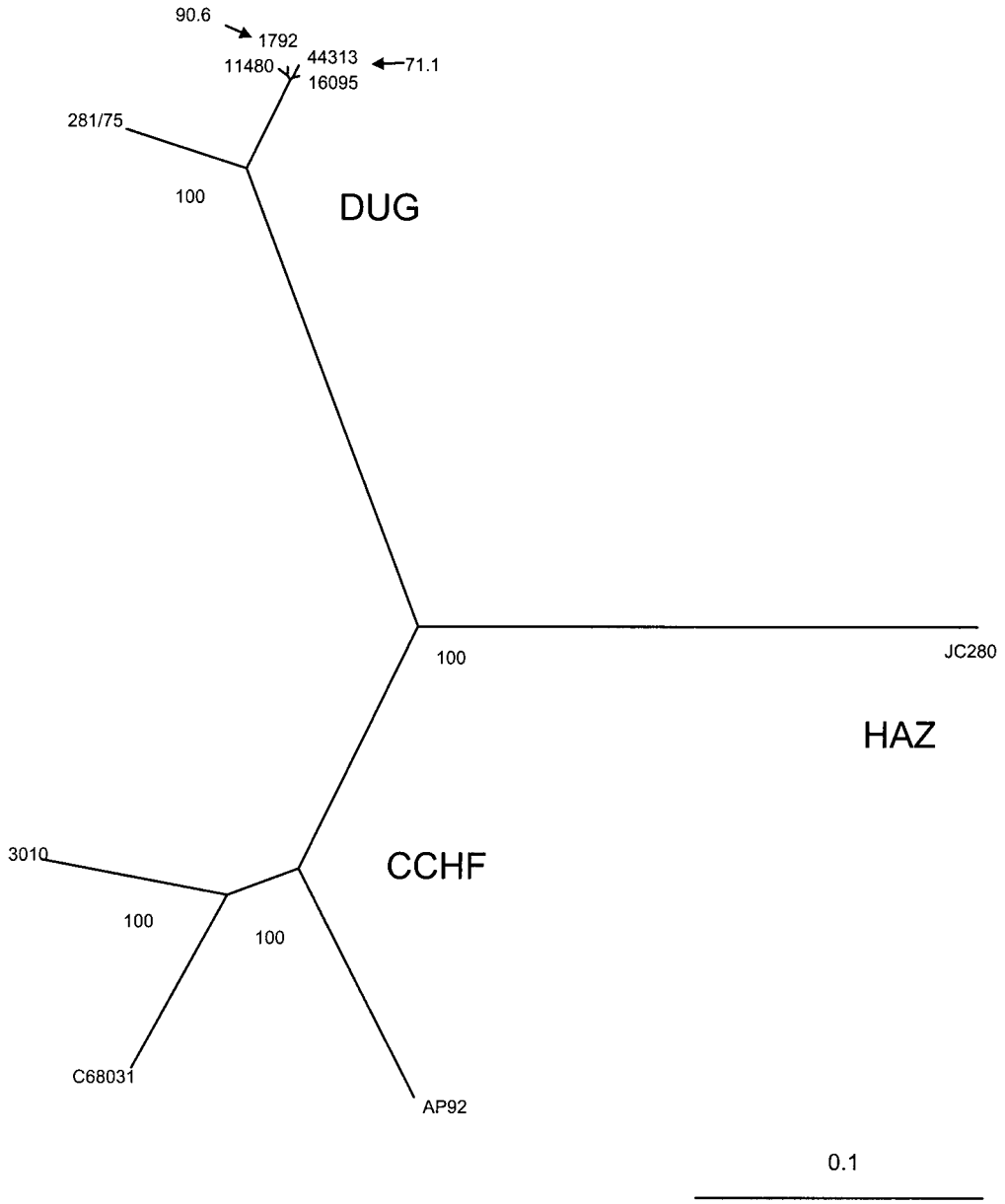

FIG. 5. Phylogenic tree deduced from the complete S segments of the following nairoviruses: DUG isolates ArD 44313, ArD 16095, IbH 11480, IbAr 1792, and KT 281/75 (this paper), CCHF isolates -C68031 (GenBank Accession No. M86625), -AP92 (U04958), and -3010 (U88416), and the Hazara JC280 isolate (M86624). Sequences were aligned using ClustalX and a phylogenetic tree generated using the neighbour-joining method of Saitou and Nei (1987) included with the ClustalX package. One thousand replicates of the sequence data were made and the percentage bootstrap support for each branch point is indicated. The tree was drawn using TreeView (Page, 1996). The scale bar represents 0.1 substitutions per base.

CAGATCAA) and digested with RNase H prior to amplification. PCR amplifications were made using PfuTurbo DNA polymerase (Stratagene). Complete S segment cDNAs of the five DUG isolates were amplified as two overlapping fragments: primer pair DgS1a (5′ TCTCAAAGACAAACGTGC) and DgS5 (5′ TGCAACAACTGGATGTGTGA) amplified nts 1–513, while primer pair DgS9 and DgS8b (5′ TCTCAAAGAGATCGTTACC) amplified nts 52–1716. The ArD 44313 DgS9–DgS8b PCR product was phosphorylated and cloned into *Eco*RV digested and dephosphorylated pBluescript SK+ vector. The N ORF of ArD 44313 was amplified using primer DgS9 and either DgS10 (5′ GACAGCTCGAGTCAAAGCCAGATTTCTGGA) or DgS10a (5′ GACAGCTCGAGTTTAGATGATGTTAACA) between nts 52–1379 or 52–1503 of the DUGS sequence presented in this paper, respectively. *Bbs*I/*Xho*I-digested products (the restriction endonuclease recognition sites in the primers are underlined) were ligated into *Nco*I/*Xho*I-cut pTM1 vector for expression of the DUG N ORF. 5′-RACE analysis (Frohman *et al.,* 1988) was performed on ArD 44313 RNA prepared as above, using the Roche 5′/3′-RACE kit as instructed by the manufacturer; the

DUG sequence-specific primers used for this experiment were DgS9 and DgS4 (5′ GAATGAGTGAAATGGGTGCA).

Additional DUG clones

Two ArD 44313 DUG S clones, kindly provided by Professor P. Nuttall, were sequenced in addition to those we constructed: clone PCRDUG8 covered nts 1–300 and clone pGM1636 extended from nt 44 to 1682.

Sequence analysis

Sequencing was performed on PCR products purified with High Prime spin-columns (Roche) or on cloned cDNAs using an automated sequence apparatus and analysed using the Wisconsin GCG series of programs running on a Unix system (Devereux *et al.,* 1984). Sequences were aligned using ClustalX (Thompson *et al.,* 1997) and phylogenetic analysis was performed with the neighbour-joining method of Saitou and Nei (1987). The phylogenetic tree was drawn using TreeView (Page, 1996). Additional analysis was performed using the PHYLIP package, version 3.5 (Felsenstein, 1993). All analyses were performed using default parameters.

Radiolabelling of cells and *in vitro* transcription–translation assays

In vivo labelling of virus–infected cells was performed as described previously (Bridgen and Elliott, 1996). *In vitro* transcription-translation reactions (25 μl) were performed using the Promega TNT-Quick reagents and 30 μCi [^{35}S]methionine.

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REFERENCES

Booth, T. F., Marriott, A. C., Stelle, G. M., and Nuttall, P. A. (1990). Dugbe virus in ticks: Histological localization studies using light and electron microscopy. *Arch. Virol*. (Suppl. 1), 207–218.

Bridgen, A., and Elliott, R. M. (1996). Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proc. Natl. Acad. Sci. USA* **93,** 15400–15404.

Burt, F. J., Spencer, D. C., Leman, P. A., Patterson, B., and Swanepoel, R. (1996). Investigation of tick-borne viruses as pathogens of humans in South Africa and evidence of Dugbe virus infection in a patient with prolonged thrombocytopenia. *Epidemiol. Infect.* **116,** 353–361.

Burt, F. J., Leman, P. A., Smith, J. F., and Swanepoel, R. (1998). The use of a reverse transcription-polymerase chain reaction for the detection of viral nucleic acid in the diagnosis of Crimean–Congo haemorrhagic fever. *J. Virol. Methods* **70,** 129–137.

Calisher, C. H. (1996). History, classification, and taxonomy of viruses in the family *Bunyaviridae*. In "The *Bunyaviridae* " (R. M. Elliott, Ed.), pp. 1–18. Plenum Press, New York.

Casals, J., and Tignor, G. H. (1980). The *Nairovirus* genus: Serological relationships. *Intervirology* **14,** 144–147.

Cash, P. (1985). Polypeptide synthesis of Dugbe virus, a member of the *Nairovirus* genus of the *Bunyaviridae. J. Gen. Virol.* **66,** 141–148.

Clerx, J. P. M., Casals, J., and Bishop, D. H. L (1981). Structural characteristics of Nairoviruses (Genus *Nairovirus*, *Bunyaviridae*). *J. Gen. Virol.* **55,** 165–178.

Clerx-van Haaster, C. M., Clerx, J. P. M., Ushima, H., Akashi, H., Fuller, F., and Bishop, D. H. L. (1982). The 3′ terminal RNA sequences of bunyaviruses and nairoviruses (*Bunyaviridae*): evidence of end sequence generic differences within the virus family. *J. Gen. Virol.* **61,** 289–292.

David-West, T. S., and Porterfield, J. S. (1974). Dugbe virus: A tick-borne arbovirus from Nigeria. *J. Gen. Virol.* **23,** 297–307.

Davies, F. G. (1997). Nairobi sheep disease. *Parassitologia* **39,** 95–98.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programmes for the VAX. *Nucleic Acids Res.* **12,** 387–395.

Elliott, R. M. (1990). Review article. Molecular biology of the *Bunyaviridae. J. Gen. Virol.* **71,** 501–522.

Elliott, R. M., Ed. (1996). "The *Bunyaviridae* " Plenum Press, New York.

Elliott, R. M. (1997). Emerging viruses: The *Bunyaviridae*. *Mol. Med.* **3,** 572–577.

Elliott, R. M., Bouloy, M., Calisher, C. H., Goldbach, R., Moyer, J. T., Nichol, S. T., Pettersson, R., Plyusnin, A., and Schmaljohn, C. S. (2000). *Bunyaviridae*. In "Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses" (M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner, Eds.), pp. 599–621. Academic Press, San Diego, CA.

Felsenstein, J. (1993). PHYLIP, Phylogeny Interference Package version 3.5; Department of Genetics, University of Washington, Seattle, WA.

Fisher-Hoch, S. P., Khan, J. A., Rehman, S., Mirza, S., Khurshid, M., and McCormick, J. B. (1995). Crimean Congo-haemorrhagic fever treated with oral ribavirin. *Lancet* **19,** 472–475.

Frohman, M. A., Dush, M. K., and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA.* **85,** 8998–9002.

Jin, H., and Elliott, R. M. (1993). Non-viral sequences at the 5′ ends of Dugbe nairovirus S mRNAs. *J. Gen. Virol.* **74,** 2293–2297.

Marriott, A. C., and Nuttall, P. A. (1992). Comparison of the S RNA segments and nucleoprotein sequences of Crimean-Congo Hemorrhagic fever, Hazara and Dugbe viruses. *Virol*ogy **189,** 795–799.

Marriott, A. C., and Nuttall, P. A. (1996a). Large RNA segment of Dugbe nairovirus encodes the putative RNA polymerase. *J. Gen. Virol.* **77,** 1775–1780.

Marriott, A. C., and Nuttall, P. A. (1996b). Molecular biology of nairoviruses. In "The *Bunyaviridae* " (R. M. Elliott, Ed.), pp. 91–104. Plenum Press, New York.

Marriott, A. C., El-Ghorr, A. A., and Nuttall, P. A. (1992). Dugbe nairovirus M RNA: nucleotide sequence and coding strategy. *Virol*ogy **190,** 606–615.

Marriott A. C., Polyzoni T., Antoniadis A., and Nuttall P. A. (1994). Detection of human antibodies to Crimean–Congo haemorrhagic fever virus using expressed viral nucleocapsid protein. *J. Gen. Virol.* **75,** 2157–2161.

Page, R. D. M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12,** 357–358.

Saitou, N., and Nei, M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4,** 406–425.

Swanepoel, R., Shepherd, A. J., Leman, P. A., Shepherd, S. P., McGilli-

vray, G. M, Erasmus, M. J., Searle, L. A, and Gill, D. E (1987). Epidemiologic and clinical features of Crimean–Congo hemorrhagic fever in southern Africa. *Am. J. Trop. Med. Hyg.* **36,**120–132.

Sweet, C., and Coates, D. (1990). Studies of the pathogenesis of Dugbe virus in normal and immunosuppressed mice. *Arch. Virol*. (Suppl. 1), 181–196.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25,** 4876–4882.

Ward, V. K., Marriott, A. C., El-Ghorr, A. A., and Nutttall, P. A. (1990). Coding strategy of the S RNA segment of Dugbe virus (*Nairovirus*; *Bunyaviridae*). *Virology* **175,** 518–524.

Ward, V. K., Marriott, A. C., Polyzoni, T., El-Ghorr, A. A., Antoniadis, A., and Nutttall, P. A. (1992). Expression of the nucleocapsid protein of Dugbe virus and antigenic cross-reactions with other nairoviruses. *Virus Res.* **24,** 223–229.

Watret, G. E., Pringle, C. R., and Elliott, R. M. (1985). Synthesis of bunyavirus-specific proteins in a continuous cell line (XTC-2) derived from *Xenopus laevis*. *J. Gen. Virol.* **66,** 473–482.