Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Identification of a New Genotype of African Swine Fever Virus in Domestic Pigs from Ethiopia

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Summary

African swine fever (ASF) is an important emerging transboundary animal disease (TAD), which currently has an impact on many countries in Africa, Eastern Europe, the Caucasus and the Russian Federation. The current situation in Europe shows the ability of the virus to rapidly spread, which stands to threaten the global swine industry. At present, there is no viable vaccine to minimize spread of the disease and stamping out is the main source of control. In February 2011, Ethiopia had reported its first suspected outbreaks of ASF. Genomic analyses of the collected ASF virus (ASFV) strains were undertaken using 23 tissue samples collected from domestic swine in Ethiopia from 2011 to 2014. The analysis of Ethiopian ASFVs partial p72 gene sequence showed the identification of a new genotype, genotype XXIII, that shares a common ancestor with genotypes IX and X, which comprise isolates circulating in Eastern African countries and the Republic of Congo. Analysis of the p54 gene also followed the p72 pattern and the deduced amino acid sequence of the central variable region (CVR) of the B602L gene showed novel tetramer repeats not previously characterized.

Introduction

African swine fever (ASF) is a devastating haemorrhagic disease of swine that ranges in severity from subacute to highly lethal acute disease depending on the doses and viral isolate involved (Sánchez-Vizcaíno et al., 2015). It is caused by African swine fever virus (ASFV), the only DNA arbovirus that consists of a large, linear, enveloped and double-stranded DNA between 170 and 190 kb in size and makes up the family *Asfarviridae* in the *Asfivirus* genus (Dixon et al., 2000).

ASF is currently endemic in sub-Saharan Africa and in Sardinia (Italy). Since 2007, multiple countries of eastern Europe, the Caucasus and the Russian Federation have been affected by the introduction of ASF from East Africa (Rowlands et al., 2008; FAO 2013). Within the European Union (EU), Lithuania and Poland made the first notifications of ASF cases in wild boar in early 2014 (Gallardo et al., 2014). ASFV has since spread in Estonia, Latvia, Lithuania and Poland with both domestic pigs and wild boar being equally affected (EFSA 2015). In sub-Saharan Africa, the number of countries reporting ASF outbreaks

has significantly grown in the last decade with currently more than 25 African countries infected with ASFV (Penrith et al., 2013). This was clearly manifested with the recent re-emergence of ASFV in 2014 and 2015 in the Ivory Coast and on Cape Verde, respectively, after over 15 years of silence (World Organisation for Animal Health (OIE) 2014; World Organisation for Animal Health (OIE) 2015a).

The risk of new introductions or reintroductions of the virus in ASF-free areas is high, taking into account the complex epidemiological situation of ASF in Africa. In eastern and southern African countries, ASFV has been maintained, for almost a century, in an ancient sylvatic cycle involving soft ticks (*Ornithodorus genus*) and asymptomatic wild African pigs, mainly warthogs (*Phacochoerus spp*), which can act as potential long term carriers allowing the virus to spill over into domestic species when the two interact. Two additional cycles have been described in endemic areas, namely a domestic pig/tick cycle, without warthog involvement, and a domestic pig/pig cycle in which the virus persists in domestic pigs in absence of vertebrate or invertebrate hosts when proper disinfection measures are not carried out (Haresnape et al., 1988; Wilkinson et al., 1988; Oura et al., 1998; Kleiboeker & Scoles 2001; Bastos et al., 2009; Penrith et al., 2004, 2013; Jori and Bastos, 2009; Jori et al.,2013; Costard et al., 2009; Gallardo et al., 2011a; Sánchez-Vizcaíno et al., 2015).

Over the last decade, the increase in pig production in Africa as well as the rapid diagnosis of the disease has led to the increased detection of ASFV in swine with a better epidemiological picture of the virus throughout infected regions. The epidemiological complexity of ASF has been clearly demonstrated in eastern and southern Africa, where genetic characterization of ASFV based on the sequencing of the C-terminal end of the major protein p72, revealed the presence of the 22 genotypes as so far identified (Boshoff et al., 2007). This rich genetic diversity is promoted through the sylvatic cycle and extended by the domestic cycle with open borders and unrestricted movement of swine in conflict areas. Several studies have assessed the role of cross border pig movements and related them to the occurrence of ASF outbreaks within the borders of several countries such as Zambia, South Africa, Mozambique, Tanzania, Kenya and Uganda (Lubisi et al., 2005; Boshoff et al., 2007; Gallardo et al., 2009, 2011a,b; Misinzo et al., 2011; Atuhaire et al., 2013). Thus, genotypes that were once thought to be specific to one country are now being detected in neighbouring countries. In contrast, western and Central Africa have traditionally shown the presence of genotype I sequences with low genetic variability and an absence of the sylvatic cycle, although transfer and dissemination of ASFV genotypes from eastern to western Africa have been demonstrated (Bastos et al., 2003; Lubisi et al., 2005; Boshoff et al., 2007; Gallardo et al., 2011a,b).

Prior to 2011, ASF had never been molecularly diagnosed in Ethiopia. On 2 January 2011, the first suspected outbreak was reported in Welkite, located in Humera in the northern Tigray region, on a small pig farm causing 100% mortality. The disease continued to spread throughout 2011 with new reported outbreaks in Tigray, Debre Zeit and Bahir Dar. This report describes the diagnosis and molecular characterization of the viruses collected during the outbreaks in Ethiopia. The genotyping strategy employed has involved standardized procedures including the sequencing of the C-terminal end of the gene encoding the p72 protein (Bastos et al., 2003) and the full-length p54 gene to place isolates into major subgroups (Gallardo et al., 2009), followed by subtype analysis of the tandem repeat sequences (TRS) located in the central variable region (CVR) of the ASFV genome (Nix et al., 2006). Collection of samples during outbreaks and surveillance efforts, as well as analysing multiple genes, is important to identify the origin of the virus during an outbreak, which increases our knowledge of what viruses are circulating and trace the spread of infection.

Materials and Methods

Study area and sampling

The rapid spread of outbreaks in early 2011 in Ethiopia alerted officials and a laboratory team from the regional veterinary laboratory and National Animal Health Diagnostic and Investigation Center (NAHDIC) investigated the outbreaks. Routine post-mortem examination of dead animals was performed, and tissue samples were collected from domestic pigs at Tigray ($n = 1$), Debre Zeit ($n = 10$) and Bahir Dar ($n = 6$). New outbreaks of suspected ASF in November 2013 led to the collection of 20 additional tissue samples (lung, spleen, lymph node, pancreas, kidney and liver) from a single farm located in Debre Zeit. These 20 samples included ten tissues collected between November and December 2013, six tissues in December 2013 and four tissues in July 2014 during a routine inspection. Finally, a total of 50 samples coming from surveillance activities conducted in January 2014 at several abattoirs located in Tatek ($n = 12$), Wollo ($n = 10$), Gondar ($n = 8$), Mojo ($n = 12$) and Debre Zeit ($n = 8$) were included in this study (Table 1). Samples were initially tested for ASFV detection at NAHDIC or the National Veterinary Institute (NVI) in Ethiopia. For further confirmation and characterization of the viruses responsible for the outbreaks, samples were sent to the IAEA, Animal Production and Health Laboratory in Seibersdorf, Austria, to the OIE reference laboratory for ASF, 'Health Surveillance Center – Animal Health Department' (VISAVET-UCM) Madrid, Spain, and to the FAO Reference Laboratory for ASF, Centro de

Table 1. Analysis of ASFV infection in domestic pigs on samples collected in Ethiopia from ASF suspected outbreaks in 2011, 2013 and 2014 and in the surveillance programme in 2014

	Origin	Date sampled	No of tissue samples collected	ASF genomic detection PCR No positives (%)
Outbreak	Humera	February 2011	1	1 (100)
Outbreak	Debre Zeit	April 2011	10	2 (20)
Outbreak	Bahir Dar	April 2011	6	4 (67)
Outbreak	Debre Zeit	November 2013–July 2014	20	14 (70)
Abattoir	Tatek	January 2014	12	0 (0)
Abattoir	Wollo	January 2014	10	0 (0)
Abattoir	Gondar	January 2014	8	2 (25)
Abattoir	Mojo	January 2014	12	0 (0)
Abattoir	Debre Zeit	January 2014	8	0 (0)

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Detection of ASFV DNA by PCR

DNA was extracted from 10% suspensions of ground tissues using the AllPrep DNA/RNA mini kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The OIE-prescribed real-time PCR assay using the ASF diagnosis primers described by King et al., 2003 (World Organisation for Animal Health (OIE), 2015b) was used to confirm the presence of ASFV DNA.

ASF molecular characterization

The molecular characterization has been performed at the IAEA APHL, Seibersdorf, Austria, the OIE reference laboratory for ASF, 'Health Surveillance Center – Animal Health Department" (VISAVET-UCM) Madrid, Spain, and in the FAO Reference Laboratory for ASF, INIA-CISA, Valdeolmos, Madrid, Spain. For genetic characterization, PCR was performed on nucleic acid extracted from ASFV selected PCR-positive samples using specific primers, which amplify three independent regions of the ASFV genome: (i) 478 bp within the 3′ end of p72 gene was amplified using primers p72-U/D (Bastos et al., 2003); (ii) the full-length gene encoding the vp54 protein was amplified using the primers PPA89/722 (Gallardo et al., 2009); (iii) the primer pairs CVR1/2 were used to amplify the central variable region (CVR) within B602L gene (Gallardo et al., 2011b). PCR products were purified using the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, USA) per manufacturers' recommendations. Nucleotide sequence of the purified products was determined using an automated 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA) or by LGC genomics (Berlin, Germany). All nucleotide sequences were deposited in GenBank (accession nos. KT795353–79 and KU291450–KU291455).

Sequence analysis

Analysis of sequence data was performed with Vector NTI 11.5 software (Life Technologies, Carlsbad CA, USA), Chromas (www.technelysium.com.au), BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html) and Clustal Omega (www.clustal.org). Two separate data sets were employed for phylogenetic analyses conducted using the MEGAv.6 software (Tamura et al., 2013): (i) A p72-gene data set comprising 234 taxa representatives of the 22 p72 ASFV described genotypes and (ii) A p54-gene data set comprising 122 taxa corresponding to East Africa ASF viruses. Neighbour joining (NJ) and minimum evolution (ME) p72 and p54 trees were constructed employing the p-distance nucleotide substitution model as implemented in the MEGA v.6 program. To determine the degree of statistical support for each node in the resulting trees, data were resampled 1000 times using the bootstrap method. To analyse the tetrameric tandem amino acid repeat sequences within the CVR of the B602L gene, deduced amino acid sequences were manually aligned with the insertion of gaps to create a more optimized alignment. Comparisons were made utilizing previous tandem repeat sequences (TRS) as described (Nix et al., 2006; Boshoff et al., 2007; Misinzo et al., 2011, 2014).

Results

Epidemiological, clinical and laboratory investigation

The first case of ASF based on clinical–pathological signs presented on 2 January 2011 in Humera, in the Tigray region, on a small pig farm affecting 100% of the population. The second outbreak located also in the Tigray region emerged a few weeks after the index case in another commercial farm and resulted in losses of 47% (119/430) mortality and 58% (252/430) morbidity. The disease affected the 5- to 10-month-old domestic pig population and was characterized by fever of 42°C, dyspnoea, nasal discharge and anorexia that lasted for 2–3 weeks. Upon necropsy, animals showed enlargement of lung, liver and lymph nodes with haemorrhage of the liver, kidney and other organs. In April 2011, subsequent ASF outbreaks were then notified in Debre Zeit and Bahir Dar in farms with intensive farming systems where pigs were confined and had no potential interaction with wildlife. Affected animals presented an acute form of ASF with anorexia, cyanotic skin, grunting and depression prior to death.

From November to December 2013, suspicions of a haemorrhagic disease were notified in a farm located in

Debre Zeit, 44 km south-east of the capital Addis Ababa, a region with a large pig population as compared with other livestock. The farm raised a total of 200 sows and 600 piglets and is a farrow-to-finish farm that involves breeding and farrowing sows as well as boars. Although gilts usually come from internal replacement, this farm eventually receives sows and boars from a farm located in Humera, in northern Ethiopia, where the first official outbreak of the disease occurred in 2011. The tissue samples were collected from swine reared on extensive to intensive farming systems under free range to semiconfined conditions with no suspected contact with wildlife.

Of the 37 samples obtained from domestic pigs located in the affected farms, 21 were ASFV positive by the OIE-prescribed real-time PCR including 7 from 2011 (1 from Humera, 2 from Debre Zeit, 4 from Bahir Dar) and 14 from 2013 in Debre Zeit. Therefore, the presence of the disease was confirmed within each of the locations affected. In addition, a positive PCR result was obtained in two domestic pigs collected from the Gondar abattoir in 2014 within the routine surveillance programme. It is important to point out that the 50 samples collected in 2014 were taken from domestic pigs with no reported illness (Table 1).

Molecular characterization of ASFV Ethiopia isolates

After the presence of ASFV was confirmed in Ethiopia, for ASFV genotyping purposes, eleven ASFV positive samples were selected from each of the outbreaks and one abattoir (Table 2). Comparisons of 405 nucleotides of the p72 sequences among Ethiopia ASFV isolates revealed a level of identity of 99.25%, with three nucleotide changes at positions 102, 105 and 336 which resulted in no amino acid changes (data not shown). To place the ASF viruses sequenced in one of the 22 p72 genotypes as so far described, the sequences obtained were compared with 234 homologous sequences representatives of each p72 described genotypes. A minimum evolution tree mapped the 11 ASFV Ethiopia isolates in a new p72 genotype, named genotype XXIII, with a high bootstrap value (95%). Genotype XXIII shares a common ancestor with genotypes IX and X, which contain East African isolates from Kenya, Uganda, Burundi and Tanzania and the Central African ASFV isolate from the Republic of Congo (Table 3). Within genotype XXIII, the Ethiopia ASF viruses were split into two branches: branch A containing the ASF viruses obtained from the 2011 outbreaks occurring in Humera and Debre Zeit and named ETH/AA, ETH/1 and ETH/3, and branch B with the viruses collected from the 2011 samples from Bahir Dar and Debre Zeit (ETH/1a, ETH/2a, ETH/3a, ETH/5a), the 2013 samples (ET13/1504 and ET13/1505) from Debre Zeit and the 2014 ASF viruses obtained from healthy domestic pigs in the Gondar abattoir named ETH/04 and ETH/017 (Fig. 1).

When comparing the 405 nt C-terminal region from the Ethiopia ASFV isolates characterized in this study with representative viruses of each of the XXII genotypes, it was found that the percentage of nucleotide variation ranged between 5.2% (21–22 changes) and 2.5% (9–10 changes). The lower amino acid diversity was found between the Ethiopia viruses and genotype IX viruses (0.7%) whereas maximum divergence was reported with genotype XIII (5.2%) (Table 4).

The value of p54 gene sequencing as additional, intermediate-resolution methods for typing of ASFV viruses has been widely demonstrated (Rowlands et al., 2008; Gallardo et al., 2009). The comparative sequence analysis of p54 PCR products obtained from the 11 ASFV Ethiopia isolates produced similar results to those obtained using p72, in that the Ethiopia viruses were split into two branches within a separate p54 genotype closely related with the p54 genotype IX and X previously described by Gallardo et al., 2009, 2011a,b; (Fig. 2).

Table 2. Ethiopia ASFV isolates selected for genotyping purposes obtained from domestic pigs whose nucleotide sequence was determined at three loci directly from tissue samples

Isolate name	Sampling localization	Date sampled	p72 gene GenBank accession no.	P54 gene GenBank accession no.	CVR GenBank accession no.
ETH/1	Debre Zeit farm	2011	KT795354	KT795366	KT795371
ETH/3	Debre Zeit farm	2011	KT795360	KT795367	KT795372
ETH/AA	Humera farm	2011	KT795353	KT795362	KT795379
ETH/1a	Bahir Dar farm	2011	KT795359	KT795363	KT795376
ETH/2a	Bahir Dar farm	2011	KT795358	KT795364	KT795377
ETH/3a	Bahir Dar farm	2011	KT795357	KT795365	KT795373
ETH/5a	Bahir Dar farm	2011	KT795361	KT795370	KT795374
ET13/1504	Debre Zeit farm	2013	KU291454	KU291452	KU291450
ET13/1505	Debre Zeit farm	2013	KU291455	KU291453	KU291451
ETH/04	Gondar abattoir	2014	KT795356	KT795368	KT795378
ETH/17	Gondar abattoir	2014	KT795355	KT795369	KT795375

Table 3. ASFV isolates belonging to genotype IX and X selected for p72 and p54 genotyping purposes obtained from ticks (Tk), domestic pigs (DP) and Warthog (WH) in Uganda (1964–2013), Tanzania (1968–2009), Kenya (1959–2013), Burundi (1984–1990) and the Republic of Congo 2009

ASFV isolate	Country	Host	Year	Sampling localization		GB Acc. No.	p72 genotype	p72_reference
Ug03H.1	Uganda	DP	2003	Hoima	Western	FJ154428	IX	Gallardo et al. (2009)
Ug03H.2	Uganda	DP	2003	Hoima	Western	FJ154429	IX	Gallardo et al. (2009)
Ug03H.3	Uganda	DP	2003	Hoima	Western	FJ154430	IX	Gallardo et al. (2009)
Ug03P.4	Uganda	DP	2003	Pallisa	Eastern	FJ154431	IX	Gallardo et al. (2009)
Ug03P.5	Uganda	DP	2003	Pallisa	Eastern	FJ154432	IX	Gallardo et al. (2009)
Ug03P.6	Uganda	DP	2003	Pallisa	Eastern	FJ154433	IX	Gallardo et al. (2009)
UGA2003/1	Uganda	DP	2003	Masaka	Central	AY351564	IX	Lubisi et al. (2005)
Ken06.B1	Kenya	DP	2006	Busia	Western	FJ154434	IX	Gallardo et al. (2009)
Ken06.B2	Kenya	DP	2006	Busia	Western	FJ154435	IX	Gallardo et al. (2009)
Ken06.B3	Kenya	DP	2006	Busia	Western	FJ154436	IX	Gallardo et al. (2009)
Ken06.B4	Kenya	DP	2006	Busia	Western	FJ154437	IX	Gallardo et al. (2009)
Ken06.B5	Kenya	DP	2006	Busia	Western	FJ154438	IX	Gallardo et al. (2009)
Ken06.Bus	Kenya	DP	2006	Busia	Western	FJ154439	IX	Gallardo et al. (2009)
Ken06.Kis	Kenya	DP	2006	Kisumu	Nyanza	FJ154440	IX	Gallardo et al. (2009)
Ken07.Eld1	Kenya	DP	2007	Uasin Gishu	Rift Valley	FJ154441	IX	Gallardo et al. (2009)
Ken07.Eld2	Kenya	DP	2007	Uasin Gishu	Rift Valley	FJ154442	IX	Gallardo et al. (2009)
Ken07.Kia	Kenya	DP	2007	Kiambu	Central	FJ154443	IX	Gallardo et al. (2009)
Ken07.Nak	Kenya	DP	2007	Nakuru	Rift Valley	FJ154444	IX	Gallardo et al. (2009)
UG07.Wak1	Uganda	DP	2007	Wakiso	Central	GQ477138	IX	Gallardo et al. (2011a)
UG07.Wak2	Uganda	DP	2007	Wakiso	Central	GQ477139	IX	Gallardo et al. (2011a)
UG07.Wak3	Uganda	DP	2007	Wakiso	Central	GQ477140	IX	Gallardo et al. (2011a)
UG07.Wak4	Uganda	DP	2007	Wakiso	Central	GQ477141	IX	Gallardo et al. (2011a)
UG07.Mukono	Uganda	DP	2007	Mukono	Central	GQ477142	IX	Gallardo et al. (2011a)
UG07.F7	Uganda	DP	2007	Nakasangola	Central	GQ477143	IX	Gallardo et al. (2011a)
UG07.F8	Uganda	DP	2007	Nakasangola	Central	GQ477144	IX	Gallardo et al. (2011a)
Ken08WH/4	Kenya	WH	2008	Machakos	Central	HM745285	IX	Gallardo et al. (2011a)
Ken08WH/5	Kenya	WH	2008	Machakos	Central	HM745286	IX	Gallardo et al. (2011a)
Ken08WH/8	Kenya	WH	2008	Machakos	Central	HM745287	IX	Gallardo et al. (2011a)
CON09/Pk45	Rep. Congo	DP	2009	Pool	Southeastern	HQ645944	IX	Gallardo et al. (2011a)
CON09/Bzz020	Rep. Congo	DP	2009	Pool	Southeastern	HQ645945	IX	Gallardo et al. (2011a)
CON09/Abo	Rep. Congo	DP	2009	Abo, Cuvette	Eastern	HQ645946	IX	Gallardo et al. (2011a)
Ken10/KakFA1	Kenya	DP	2010	Kakamega	Western	KC112561	IX	Unpublished INIA-CISA
Ken10/Kis027	Kenya	DP	2010	Kisumu	Nyanza	KC112562	IX	Unpublished INIA-CISA
Ken10/Kis028	Kenya	DP	2010	Kisumu	Nyanza	KC112563	IX	Unpublished INIA-CISA
Ug10.Amuru	Uganda	DP	2010	Amuru	Northern	KC990902	IX	Atuhaire et al. (2013)
Ug10.Moyo2	Uganda	DP	2010	Moyo	Northern	KC990898	IX	Atuhaire et al. (2013)
Ug10.Moyo1	Uganda	DP	2010	Moyo	Northern	KC990897	IX	Atuhaire et al. (2013)
Ug10.Tororo	Uganda	DP	2010	Tororo	Eastern	KC990896	IX	Atuhaire et al. (2013)
Ug10.Adjumani	Uganda	DP	2010	Adjumani	Northern	KC990894	IX	Atuhaire et al. (2013)
Ug10.Kumi	Uganda	DP	2010	Kumi	Eastern	KC990892	IX	Atuhaire et al. (2013)
Ken11/Bus1.2	Kenya	DP	2011	Busia	Western	KC112564	IX	Unpublished INIA-CISA
Ken11/Kia2.1	Kenya	DP	2011	Kiambu	Western	KC112565	IX	Unpublished INIA-CISA
Ken11/ThikP06	Kenya	DP	2011	Thika	Central	KC112566	IX	Unpublished INIA-CISA
Ken11/KakSP	Kenya	DP	2011	Kakamega	Western	KC112567	IX	Unpublished INIA-CISA
Ken11/Bum002	Kenya	DP	2011	Bumula	Western		IX	Unpublished INIA-CISA
Ken11/KisP1	Kenya	DP	2011	Kisumu	Nyanza		IX	Unpublished INIA-CISA
Ken11/KerP27	Kenya	DP	2011	Kericho	Rift Valley		IX	Unpublished INIA-CISA
Ken11/NakP29	Kenya	DP	2011	Nakuru	Rift Valley		IX	Unpublished INIA-CISA
Ken11/KiaP31	Kenya	DP	2011	Kiambu	Central		IX	Unpublished INIA-CISA
Ken11/KisP52	Kenya	DP	2011	Kisumu	Nyanza		IX	Unpublished INIA-CISA
Ken11/Thikp49	Kenya	DP	2011	Thika	Central		IX	Unpublished INIA-CISA
Ken11/Kilifili	Kenya	DP	2011	Kilifi	Coast		IX	Unpublished INIA-CISA
Ug11.Mpigi	Uganda	DP	2011	Mpigi	Central	KC990895	IX	Atuhaire et al. (2013)
Ug11.Kampala2	Uganda	DP	2011	Kampala	Central	KC990893	IX	Atuhaire et al. (2013)
Ken11/kisauni2	Kenya	DP	2011	Mombasa	Coast	KJ626192	IX	Unpublished ILRI

Table 3. (continued)

ASFV isolate	Country	Host	Year	Sampling localization		GB Acc. No.	p72 genotype	p72_reference
Ken11/kisauni1	Kenya	DP	2011	Mombasa	Coast	KJ626191	IX	Unpublished ILRI
Uga12.Kalungu3	Uganda	DP	2012	Kalungu	Central	KF303313	IX	Atuhaire et al. (2013)
Uga12.Kalungu2	Uganda	DP	2012	Kalungu	Central	KF303312	IX	Atuhaire et al. (2013)
Uga12.Kalungu1	Uganda	DP	2012	Kalungu	Central	KF303311	IX	Atuhaire et al. (2013)
Uga12.Nakasongola	Uganda	DP	2012	Nakasangola	Central	KF303310	IX	Atuhaire et al. (2013)
Ug12.Kampala4	Uganda	DP	2012	Kampala	Central	KC990904	IX	Atuhaire et al. (2013)
Ug12.Kyenjojo	Uganda	DP	2012	Kyenjojo	Western	KC990903	IX	Atuhaire et al. (2013)
Ug12.Wakiso	Uganda	DP	2012	Wakiso	Central	KC990901	IX	Atuhaire et al. (2013)
Ug12.Kampala3	Uganda	DP	2012	Kampala	Central	KC990900	IX	Atuhaire et al. (2013)
Ug12.Lira	Uganda	DP	2012	Lira	Northern	KC990899	IX	Atuhaire et al. (2013)
Ug12.Kabale1	Uganda	DP	2012	Kabale	Western	KC990890	IX	Atuhaire et al. (2013)
Uga12.Lango3	Uganda	DP	2012	Lango	Northern	KF303321	IX	Atuhaire et al. (2013)
Uga12.Lango2	Uganda	DP	2012	Lango	Northern	KF303320	IX	Atuhaire et al. (2013)
Uga12.Lango1	Uganda	DP	2012	Lango	Northern	KF303319	IX	Atuhaire et al. (2013)
Uga12.Busoga2	Uganda	DP	2012	Busoga	Eastern	KF303318	IX	Atuhaire et al. (2013)
Uga12.Busoga1	Uganda	DP	2012	Busoga	Eastern	KF303317	IX	Atuhaire et al. (2013)
Uga12.Nakaseke	Uganda	DP	2012	Nakaseke	Central	KF303316	IX	Atuhaire et al. (2013)
Uga12.Kibaale	Uganda	DP	2012	Kibaale	Western	KF303315	IX	Atuhaire et al. (2013)
Uga12.Sembabule	Uganda	DP	2012	Sembabule	Central	KF303314	IX	Atuhaire et al. (2013)
Ug13.Busia2	Uganda	DP	2013	Busia	Eastern	KC990906	IX	Atuhaire et al. (2013)
Ug13.Busial	Uganda	DP	2013	Busia	Eastern	KC990905	IX	Atuhaire et al. (2013)
Ug13.Kampala1	Uganda	DP	2013	Kampala	Central	KC990891	IX	Atuhaire et al. (2013)
ken13/nakuru.1	Kenya	DP	2013	Nakuru	Rift Valley	KM000166	IX	Unpublished ILRI
ken13/kakamega.1	Kenya	DP	2013	Kakamega	Western	KM000164	IX	Unpublished ILRI
ken13/nyadorera.1	Kenya	DP	2013	Nyanza	Western	KM000163	IX	Unpublished ILRI
ken13/busia.9	Kenya	DP	2013	Busia	Western	KM000162	IX	Unpublished ILRI
MWHOG/9	Kenya	WH	1959	Rift Valley	Rift Valley	AY351565	X	Lubisi et al. (2005)
Ug64	Uganda	DP	1964	NK		FJI74383	X	Gallardo et al. (2009)
TAN/Kwh12	Tanzania	WH	1968	Serengeti National Park		AF301546	X	Lubisi et al. (2005)
BUR/2/84	Burundi	DP	1984	NK		AF449464	X	Bastos et al. (2003)
BUR/1/84	Burundi	DP	1984	NK		AF449463	X	Bastos et al. (2003)
KIRW/891	Tanzania	WH	1989	Serengeti National Park		AY351514	X	Lubisi et al. (2005)
KIRT/894	Tanzania	Tk	1989	Serengeti National Park		AY351513	X	Lubisi et al. (2005)
KIRT/893	Tanzania	Tk	1989	Serengeti National Park		AY351512	X	Lubisi et al. (2005)
KIRT/892	Tanzania	Tk	1989	Serengeti National Park		AY351511	X	Lubisi et al. (2005)
BUR/90/3	Burundi	DP	1990	Muyinga	North	AY351525	X	Lubisi et al. (2005)
BUR/90/2	Burundi	DP	1990	NK		JX467634	X	Unpublished OVI
BUR/90/1	Burundi	DP	1990	NK		AF449472	X	Bastos et al. (2003)
Ken05/Tk1	Kenya	Tk	2005	Machakos	Central	HM745253	X	Gallardo et al. (2011a)
Ken05/Tk2	Kenya	Tk	2005	Machakos	Central	HM745254	X	Gallardo et al. (2011a)
Ken05/Tk3	Kenya	Tk	2005	Machakos	Central	HM745255	X	Gallardo et al. (2011a)
Ken05/Tk4	Kenya	Tk	2005	Machakos	Central	HM745256	X	Gallardo et al. (2011a)
Ken05/Tk5	Kenya	Tk	2005	Machakos	Central	HM745257	X	Gallardo et al. (2011a)
Ken05/Tk6	Kenya	Tk	2005	Machakos	Central	HM745258	X	Gallardo et al. (2011a)
Ken05/Tk7	Kenya	Tk	2005	Machakos	Central	HM745259	X	Gallardo et al. (2011a)
Ken05/Tk8	Kenya	Tk	2005	Machakos	Central	HM745260	X	Gallardo et al. (2011a)
Ken05/Tk9	Kenya	Tk	2005	Machakos	Central	HM745261	X	Gallardo et al. (2011a)
Ken05/Tk10	Kenya	Tk	2005	Machakos	Central	HM745262	X	Gallardo et al. (2011a)
Ken05.DPk2	Kenya	DP	2005	Kiambu	Central	HM745263	X	Gallardo et al. (2011a)
Ken05.DPk16	Kenya	DP	2005	Kiambu	Central	HM745264	X	Gallardo et al. (2011a)
Ken05.DPk18	Kenya	DP	2005	Kiambu	Central	HM745265	X	Gallardo et al. (2011a)
Ken05.DPk21	Kenya	DP	2005	Kiambu	Central	HM745266	X	Gallardo et al. (2011a)
Ken05.DPk27	Kenya	DP	2005	Kiambu	Central	HM745267	X	Gallardo et al. (2011a)
Ken05.DPN2	Kenya	DP	2005	Nandi	Central	HM745268	X	Gallardo et al. (2011a)
Ken05.DPN15	Kenya	DP	2005	Nandi	Central	HM745269	X	Gallardo et al. (2011a)
Ken05.DPU1	Kenya	DP	2005	Kiambu	Central	HM745271	X	Gallardo et al. (2011a)

Table 3. (continued)

ASFV isolate	Country	Host	Year	Sampling localization		GB Acc. No.	p72 genotype	p72_reference
Ken05.DPU2	Kenya	DP	2005	Kiambu	Central	HM745272	X	Gallardo et al. (2011a)
Ken05.DPU11	Kenya	DP	2005	Kiambu	Central	HM745273	X	Gallardo et al. (2011a)
Ken05.DPU22	Kenya	DP	2005	Kiambu	Central	HM745274	X	Gallardo et al. (2011a)
Ken08Tk.2/1	Kenya	Tk	2008	Machakos	Central	HM745275	X	Gallardo et al. (2011a)
Ken08Tk.2/3	Kenya	Tk	2008	Machakos	Central	HM745276	X	Gallardo et al. (2011a)
Ken08BP/HB	Kenya	WH	2008		Nyanza	JN590911	X	Unpublished INIA-CISA
Ken08DP/Ndhiwa	Kenya	DP	2008	Ndhiwa	Nyanza	JN590912	X	Unpublished INIA-CISA
Ken08DP/Nyarongi	Kenya	DP	2008	Nyaron	Nyanza	JN590913	X	Unpublished INIA-CISA
Ken09Tk.13/1	Kenya	Tk	2009	Machakos	Central	HM745277	X	Gallardo et al. (2011a)
Ken09Tk.13/2	Kenya	Tk	2009	Machakos	Central	HM745278	X	Gallardo et al. (2011a)
Ken09Tk.15/4	Kenya	Tk	2009	Machakos	Central	HM745279	X	Gallardo et al. (2011a)
Ken09Tk.15/6	Kenya	Tk	2009	Machakos	Central	HM745280	X	Gallardo et al. (2011a)
Ken09Tk.19/2	Kenya	Tk	2009	Machakos	Central	HM745281	X	Gallardo et al. (2011a)
Ken09Tk.19/7	Kenya	Tk	2009	Machakos	Central	HM745282	X	Gallardo et al. (2011a)
Ken09Tk.19/11	Kenya	Tk	2009	Machakos	Central	HM745283	X	Gallardo et al. (2011a)
Ken09Tk.20/5	Kenya	Tk	2009	Machakos	Central	HM745284	X	Gallardo et al. (2011a)
TAN/09/Longido	Tanzania	DP	2009	Longido	Arusha	JX262383	X	Misinzo et al. (2011)
UG10/Tk3.2	Uganda	Tk	2010	Lake Mburu National Park			X	Unpublished INIA-CISA

NK, Not known.

The nature of the new genotype discovered was confirmed through the amplification of the TRS within the CVR of the B602L gene. Sequence analyses resulted in the identification of three different variants among the ASFV Ethiopia viruses (Table 5). The CVR variants 1 and 2 were characterized by the presence of three different types of amino acid tetramers (J = GTDT; K = CTSP; L = YTNT) not previously found in ASFV isolates characterized up to now. The most abundant tetrameric repeat sequence AAABJJKL (variant 1) was identified in the Ethiopia domestic pig isolates, which were responsible of the outbreaks that occurred in 2011 and 2013 in Debre Zeit as well as to that observed in the viruses recovered from the abattoir in 2014. Interestingly, although clustered within the same p72 genotype XXIII branch A, the ETH/3a and ETH/5a Ethiopia 2011 viruses from Bahir Dar contained a pattern of tetramer repeats ABNAAAAACBNABTDBNAFA previously described in ASFV isolates from Cameroon (Wade A., unpublished results). This pattern was also present in the ETH/3 virus, whereas the other two viruses, which were clustered within the p72 genotype XXIII branch B, were subtyped within variant 2 which presented a unique type of TRS related to variant 1. The results confirm the added value of analysing the TRS for deeper resolution in ASFV molecular epidemiology.

Discussion

Gaining knowledge of the molecular epidemiology of ASF virus isolates is necessary in order to develop effective prevention and control strategies at both local and regional levels to control the disease. This knowledge can help predict the possible evolution of the epidemic in the affected regions and reinforce the control measures on factors contributing to virus transmission.

To elucidate the molecular characterization and phylogeny of the ASFV field strains associated with the 2011–2014 ASF outbreaks in Humera, Bahir Dar and Debre Zeit, 11 ASFV strains were selected for genotyping. According to the work developed by Bastos et al., 2003; any two viruses from distinct p72 genotypes must differ from each other at a minimum of four nucleotide sites across the 405 nt C-terminal region used for p72 genotyping purposes. The Ethiopia strains fulfilled these criteria thus adding another genotype to the known 22 ASFV genotypes. The highest nucleotide identity of the Ethiopia viruses was found with p72 genotype IX (Table 4). This genotype comprises ASFV isolates obtained mainly from domestic pig outbreaks in Kenya, Uganda and the Republic of Congo since 2003 until 2013 (Lubisi et al., 2005; Gallardo et al., 2009, 2011a,b; Atuhaire et al., 2013). Kenya and Uganda share borders to the south of Ethiopia suggesting that the ASFV Ethiopia strains might have evolved from the same origin although the mechanisms of the evolution of the viruses are yet unknown. Possibly, the ancestor of genotype IX, the closely related genotype X and the newly identified genotype XXIII was present somewhere in East Africa. Favourable conditions for the adaptation of genotypes IX and X allowed these genotypes to thrive and transmit in Kenya and Uganda while genotype XXIII remained undiscovered.

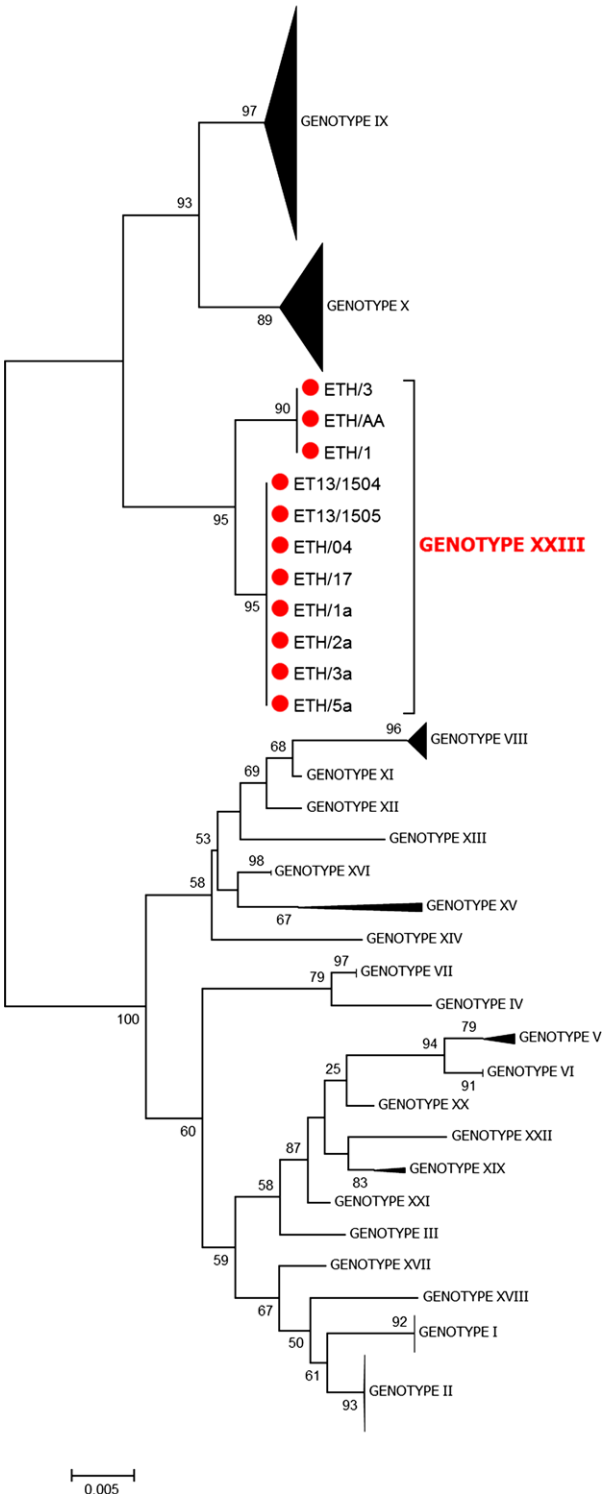

Fig. 1. Minimum evolution phylogenetic tree of the Ethiopia ASFV isolates based on the analysis of the 405 nucleotides situated at the C-terminal end of the p72 coding gene relative to the 22 p72 genotypes (labelled I-XXII), including 234 nucleotide sequences. The tree was inferred using the minimum evolution (ME) method following initial application of a neighbour-joining algorithm. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The percentage of replicate trees >50% in which the associated taxa clustered together by bootstrap analysis (1000 replicates) is shown adjacent to the nodes. The robustness of the ME tree was tested using the close-neighbour-interchange (CNI) algorithm at a search level of 1. ASFV Ethiopia isolates genotyped in this study are marked in red (●) within the genotype XXIII red labelled.

Table 4. Nucleotide (NT) and amino acid (AA) variation across the 405 nt and 135 aa C-terminal region of p72 protein between the Ethiopia ASFV isolates placed into the genotype 23 (branches A and B) and each of the 22 p72 genotypes

	P72 genotype 23 branch 1				P72 genotype 23 branch 2			
	NT variation		AA variation		NT variation		AA variation	
ASFV genotypes	No.	%	No.	%	No.	%	No.	%
9	10	2.5	1	0.7	9	2.2	1	0.7
10	16	4.0	3	2.2	15	3.7	3	2.2
8	22	5.4	4	3.0	23	5.7	4	3.0
11	18	4.4	4	3.0	19	4.7	4	3.0
12	18	4.4	4	3.0	19	4.7	4	3.0
14	20	4.9	4	3.0	21	5.2	4	3.0
15	21	5.2	4	3.0	22	5.4	4	3.0
16	17	4.2	4	3.0	18	4.4	4	3.0
17	19	4.7	4	3.0	20	4.9	4	3.0
21	17	4.2	4	3.0	18	4.4	4	3.0
1	22	5.4	5	3.7	23	5.7	5	3.7
2	20	4.9	5	3.7	21	5.2	5	3.7
5	23	5.7	5	3.7	24	5.9	5	3.7
6	22	5.4	5	3.7	23	5.7	5	3.7
18	22	5.4	5	3.7	23	5.7	5	3.7
19	19	4.7	5	3.7	20	4.9	5	3.7
20	19	4.7	5	3.7	20	4.9	5	3.7
3	20	4.9	6	4.4	21	5.2	6	4.4
4	20	4.9	6	4.4	21	5.2	6	4.4
7	20	4.9	6	4.4	21	5.2	6	4.4
22	21	5.2	6	4.4	22	5.4	6	4.4
13	21	5.2	7	5.2	22	5.4	7	5.2

Genotype XXIII could have maintained a silent presence in ticks and warthogs in Ethiopia until the increase in domestic pig production in recent years allowed for the movement into susceptible pig breeds within Ethiopia.

Further sequence analysis of ASFV isolates obtained during the epidemic cases that occurred in 2013 at the Debre Zeit farm, epidemiologically linked them with the index case from 2011.

The results obtained throughout the p72 genotyping were confirmed by the full sequencing of the p54 gene. In contrast, the partial analysis of the hyper variable region located within

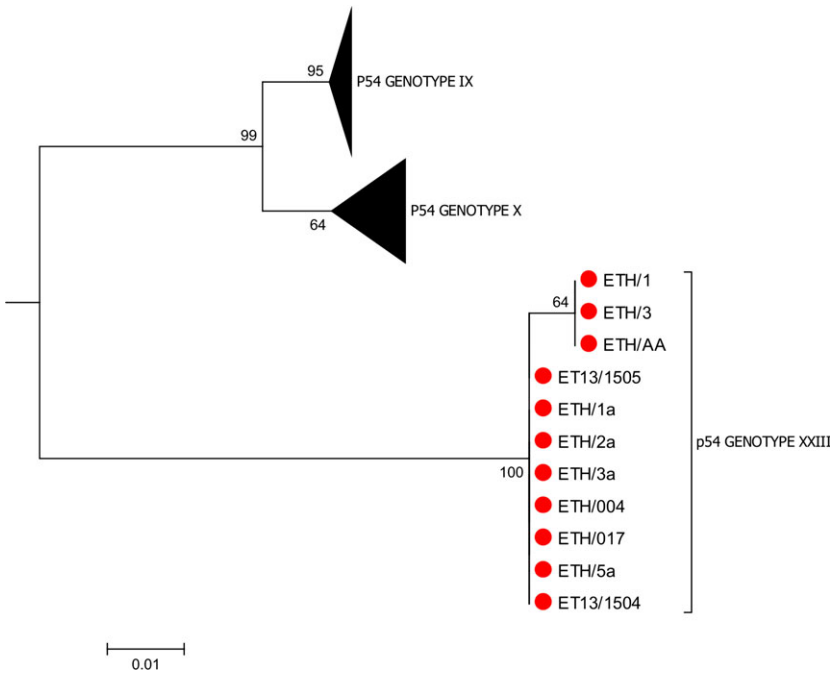

Fig. 2. Subtree depicting the p54 subtype X and IX of the full-length p54 gene (P54) generated using sequences from 122 East African ASFV isolates including the Ethiopia viruses analysed in this study. The evolutionary history was inferred using the minimum evolution method (ME). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ME trees were further analysed using the close-neighbour-interchange (CNI) algorithm at a search level of 1. A neighbour-joining algorithm was used to generate the initial trees using 1000 replicates. Bootstrap values > 50% are indicated next to the relevant node. (●) indicates ASF viruses characterized in this study.

Table 5. Amino acid sequence of the tetrameric repeats that constitute the central variable region (CVR) of the *B602L* gene identified in the Ethiopia viruses belonging to p72 genotype XXIII. Key: A (CAST); B (CADT); C (GAST); D (CASM); F (CANT, CAAT); J (GTDT); K (CTSP); L (YTNT); T (NVNT); N (NVDT)

P72 genotype XXIII	Isolate	Collection	Date	CVR VARIANT	CVR amino acid sequence	No repeats
A	ETH/1a	outbreak	2011	1	AAABJJKL	8
A	ETH/2a	outbreak	2011	1	AAABJJKL	8
A	ET13/1504	outbreak	2013	1	AAABJJKL	8
A	ET13/1505	outbreak	2013	1	AAABJJKL	8
A	ETH/04	abattoir	2014	1	AAABJJKL	8
A	ETH/17	abattoir	2014	1	AAABJJKL	8
B	ETH/AA	outbreak	2011	2	ABBBBFFBFBJJKL	14
B	ETH/1	outbreak	2011	2	ABBBBFFBFBJJKL	14
B	ETH/3	outbreak	2011	3	ABNAAAAACBNABTDBNAFA	20
A	ETH/3a	outbreak	2011	3	ABNAAAAACBNABTDBNAFA	20
A	ETH/5a	outbreak	2011	3	ABNAAAAACBNABTDBNAFA	20

the B602L gene allowed us to identify three different CVR variants, two of which are novel in sequence further supporting the separate genotype. The similarity of variant 3 between Ethiopia and Cameroon is consistent with previous data, which reported ASFV dissemination from East to West Africa through persistently infected wild or domestic animals or by the movement of infected pigs or pork products (Gallardo et al., 2011b). It is therefore important to study ASF in Central Africa with an emphasis on countries such as Central African Republic and Chad to better understand and trace the movement of virus between countries.

Interestingly, the virus recovered from apparently healthy animals during the survey conducted in 2014 at the Gondar abattoir presented 100% of identity in the three ASFV genome regions analysed with the 2011 and 2013 ASFV strains, which induced an acute form of the disease

as it was reported in the affected farms. It should be considered that after the first introduction of the disease in a region, increased numbers of subacute and subclinical infections can occur over time and that mortality rates decline. In such situations, the clinical manifestations are more variable and recognition of the disease becomes difficult in the field, emphasizing the need of implementation of appropriate surveillance programmes to control the disease (Mebus et al., 1978; Mebus and Dardiri, 1979, 1980; Thomson et al., 1979; Hess, 1981; Wilkinson et al., 1981, 1983; Wilkinson, 1984; Sánchez-Botija, 1982; Nsalambi, 1993; Penrith et al., 2013; Gallardo et al., 2015; Sánchez-Vizcaíno et al., 2015).

There is currently no data regarding the presence of ASF in other boarder countries such as Sudan, South Sudan, Eritrea, Djibouti and Somalia. While many of these neighbouring countries, due to religious preferences, have minimal domestic swine, the sylvatic cycle between warthogs and ticks remains a possibility and thus should be investigated. As the domestic population of swine in Africa continues to grow, so does the problematic economic constraints when there is no vaccine and the only form of control is stamping out of infected pigs. This makes the need for a vaccine more imperative than ever to control this TAD. So far, all attempts to produce safe vaccines against ASFV infection have demonstrated only homologous protection (Manso-Ribeiro et al., 1963; Ruiz-Gonzalvo et al., 1996). Additionally, swine that survive ASFV infection against one genotype have shown solid immunity to challenge from a homologous strain but not usually against heterologous strains even when they are in the same genotype (Manso-Ribeiro et al., 1963; Leitão et al., 2001; King et al., 2011; Mulumba-Mfumu et al., 2015). It is therefore likely that effective control of ASF will rely on the availability of several vaccines based on the specific isolates circulating in each region. Increasing our knowledge of ASFV isolates worldwide through whole genome sequencing will aid in the development of promising research approaches such as the creation of deletion mutants based on protection against the strains of the same genotypes.

While the detection of ASFV in Ethiopia is more recent than other East African countries, the detection of a completely different genotype leaves a gap in understanding the epidemiology of the virus. Questions remain whether these strains have been circulating in the sylvatic cycle or if they were transported from another unknown location where ASF had not been detected. Continuation of surveillance is important for us to better understand the epidemiology of the virus so that a future vaccine can be prepared that protects domestic swine from future outbreaks.

The discovery of a new ASFV genotype emphasises the importance and high variability of ASFV isolates in Africa. In addition and due to the increase of viruses sequenced over the last decade, a need for reclassification of the current ASFV isolates within the p72 genotypes should be considered.

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Conflicts of interest

The authors declared no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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