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Measles Virus Strains Circulating in Ethiopia in 1998–1999: Molecular Characterisation Using Oral Fluid Samples and Identification of a New Genotype

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A measles outbreak in December 1998 in Bedelle (vaccine coverage <40%) and two sporadic cases in Addis Ababa, Ethiopia, were investigated. Paired serum and oral fluid samples were collected 2–8 days after the onset of symptoms. A total of 53 of 55 outbreak cases and both sporadic cases were positive for serum measles virus-specific IgM. Oral fluid measles-specific IgM was positive in 71% of cases collected up to 5 days after onset and in 90% collected at 6–8 days. By contrast, 100% of oral fluid samples were positive for measles virus RNA by RT-PCR, suggesting that early collection of samples favoured the detection of measles virus RNA by RT-PCR. The measles virus strain in the outbreak was identified as genotype D4. One strain from a sporadic case was also genotype D4; the strain from the other sporadic case was assigned to clade D but was distinct. The degree of divergence from recognised clade D strains suggested that, together with three strains from the United Kingdom, it represents an additional genotype of clade D (GenBank accession numbers AF280800–280807). ***J. Med. Virol. 65:373–380, 2001.***

KEY WORDS: measles outbreak; Ethiopia; new genotype

INTRODUCTION

The global burden of disease from measles was estimated in 1996 to be 36.5 million cases and 1 million deaths [WHO, 1994; Strebel, 1998]. The disease is responsible for 10% of mortality from all causes among children <5 years of age and it is the eighth leading cause of death worldwide [WHO, 1994; Loos, 1998]. In Ethiopia, measles is the commonest cause of mortality and morbidity in children. Every year 50,000–60,000 deaths occur in children <5 years of age as a result of measles and its complications [Ministry of Health, 1998]. Although routine measles vaccination coverage has increased overall in Ethiopia from 29% in 1994 to 52% in 1997, it is still low and less than the 1997 global and African average coverage figures of 79% and 56%, respectively [Ministry of Health, 1998]. Associated with the low vaccination coverage and prevailing poor living conditions, measles outbreaks occur frequently in urban and rural areas with case fatality rates as high as 15–20% [Lintjorn, 1986].

Measles virus is a negative sense, single-stranded RNA virus classified in the *Morbillivirus* genus of the Paramyxoviridae family [Lintjorn, 1986]. Analysis of sequence data of different measles virus genotypes has been used as an epidemiological tool to investigate transmissions of measles in endemic infections and in outbreaks. Genetic analysis of strains associated with the 1994 and 1995 measles outbreaks in the United States confirmed that infections were the result of international importation of the virus [Rota et al., 1995]. Recent studies demonstrated the endemic co-circulation of two distinct measles genotypes of clade B in western and central Africa [Hanses et al., 1999], while in countries in southern Africa, genotypes of clades A and D have been identified [Kreis et al., 1997]. Apart from one report of genotype D4 from Kenya [Bellini and Rota, 1998] there is no information available on measles virus strains circulating in countries of eastern Africa such as Ethiopia. Molecular identification of the geographical origin of measles virus strains is a key strategy in achieving the aims of

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the World Health Organisation measles elimination/eradication programme [WHO, 1997].

In December 1998, a measles outbreak was reported in the regional state of Oromia in western Ethiopia, affecting three districts. An outbreak investigation was mounted in one district, Bedelle, during which paired oral fluid and serum samples were collected for measles-specific IgM serology to confirm recent measles. Oral fluids have also been used as a source of viral RNA for genetic analysis of MV strains (Jin et al., 1996, 1997], and the decision was made to use these samples to characterise the virus strain causing the outbreak. Measles virus strains detected in oral fluids from sporadic cases from Addis Ababa in 1998 were also analysed. The phylogenetic relationships of these Ethiopian strains and of others from different geographical origins were studied in order to determine the source of the outbreak and to discriminate between epidemiological related strains.

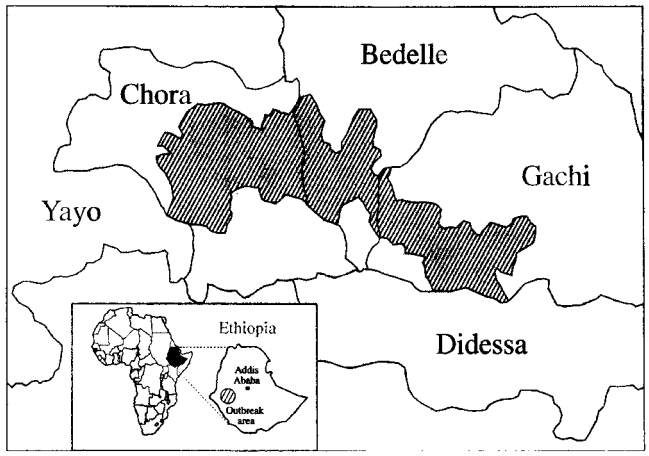

Fig. 1. Districts affected by the 1998–1999 measles outbreak in Ethiopia. The area in which measles infection occurred is highlighted.

MATERIALS AND METHODS

Study Areas and Populations

The measles outbreak occurred from December 25, 1998 to January 31, 1999 in the remote and largely rural location of Illubabor. This is one of 12 administrative zones of the Oromia regional state (population 954,352 in 1995) in western Ethiopia. The Illubabor zone consists of 12 districts with one hospital, five health centres, and 78 health stations. All health facilities undertake integrated maternal and child health activities including vaccination services for children <1 year of age and women of childbearing age. There are 57 static sites and 105 outreach teams conducting vaccinations. Reported measles vaccination coverage in the zone was low with 36% measles coverage in 1998. Over the 3-year period before the outbreak, no measles cases were reported in the Illubabor zone.

Of the 12 districts in the Illababor zone, three were affected by the 1998–1999 measles outbreak. These were Chora, Bedelle, and Gachi districts, which lie adjacent to each other between longitude 36°20′ and latitude 8°20′ and at an altitude of 1,500–2,000 m above sea level (Fig. 1). Chora district was the first to report the outbreak (December 25, 1998), followed by Bedelle and Gachi. A total of 30 villages and two towns were affected by the outbreak.

Addis Ababa (population 2.1 million in 1994) has six administrative zones (districts) with different vaccination coverage. For the target population (children aged 9 months) of 32,548, reported measles vaccination coverage was 88.5% in 1998 and has not been below 75% since records have become available (1992–1993).

Study Subjects and Specimens Examined

The study subjects were 55 patients visiting the Bedelle Health Centre and the Health Clinic in Abdella, a town located 15 km southwest of Bedelle town (Fig. 1). Acute measles patients reported by Health Post attendants (nonmedical personnel trained for notifying infectious diseases) were also traced from house to house in the different villages of Bedelle district. The clinical characteristics of the measles virus infection observed by the Health Centre doctor or nurse were recorded. Paired oral fluid and serum samples were collected from patients aged 2–8 days (mean = 4.4 days) after onset of symptoms. Oral fluid specimens collected using sponge swab collection devices [Nokes et al., 1998] were processed as previously described [Nigatu et al., 1999] in the Health Centre and transported in liquid nitrogen to Ethiopian Health and Nutrition Research Institute, Addis Ababa where they kept at −20°C. Specimens packed in polystyrene boxes with dry ice were then transported to Central Public Health Laboratory, United Kingdom, for measles IgM testing and genotyping. In addition to the outbreak samples, two paired oral fluid/serum specimens were collected from Addis Ababa during the same period in 1998–1999. One was collected from a patient diagnosed at a private clinic in zone 5 and the other from a patient at Akaki Health Centre in zone 6.

Serum Measles-Specific IgM

Measles-specific IgM in serum was detected by antibody capture enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Light Diagnostics, Chemicon Temecula, CA, USA). The Light Diagnostics measles capture ELISA uses a recombinant measles virus nucleoprotein antigen [Hummel et al., 1992].

Oral Fluid Measles-Specific IgM

Measles-specific IgM in oral fluid was determined by IgM-capture ELISA (MACELISA) using the same anti-human IgM, measles virus antigen and monoclonal antibody reagents as previously described for IgM-

capture radioimmunoassay (RIA) [Perry et al., 1993]. The MACELISA was performed with the FITC/anti-FITC amplification system as described for measles virus IgG-capture ELISA. Results were expressed as T/N ratios (test sample OD/negative control OD), which helped minimise inter-assay variation. The cutoff T/N ratio, determined by mixture model analysis [Parker et al., 1990] of results from 525 oral fluid samples collected pre- and postvaccination in a measles vaccination study, was selected to give the predicted lowest proportion of false-positive and false-negative results. T/N values of >2.0 were considered positive.

Reverse Transcription-Polymerase Chain Reaction

Nucleic acid was extracted from oral fluid specimens using the silica-guanidinium thiocyanate method and measles virus cDNA produced by reverse transcription-polymerase chain reaction (RT-PCR), as described previously [Jin et al., 1996]. The primers for amplifying and sequencing a 550- and a 450-nucleotide (nt) fragment of the hemagglutinin (H) gene, the entire H gene, and the entire nucleoprotein (N) gene have been described (Jin et al., 1996, 1997, 1998a]. In parallel with negative controls (deionised H_2O), the Loss MV strain [Synitsyna et al., 1990] was used as a positive control.

Nucleotide and Amino acid Sequence Determination

PCR amplicons were excised and purified from agarose gels before sequencing [Jin et al., 1996]. Sequencing was carried out using the Taq DyeDeoxy-terminator cycle sequencing kit (PE Applied Biosystems, Warrington, England, UK) with specific primers [Jin et al., 1996, 1997, 1998a] in an AB1373A automatic DNA sequencer. Nucleotide and deduced amino acid (aa) sequences were analysed with SeqED (version 1.0.3. program, PE Applied Biosystems). Phylogenetic trees were constructed either by the Clustal of Megalign program (DNASTAR, Madison, WI, USA) or by bootstrap analysis (1,000 times) using neighbour-joining of the PAUP 4.0 Beta 2 package (Sinauer Associate, Inc., Sunderland, MA, USA).

RESULTS

Epidemiological Findings

A total of 1,603 cases fulfilling the WHO measles case definition [Loos, 1998] were reported. The outbreak was first reported in Chora district and within 20 days spread to all 20 villages in this district. After this time the number of reported cases declined. The incidence of measles was higher in the Chora district (1,087 per 100,000) than in Bedelle (373 per 100,000) and Gachi (221 per 100,000) districts to which it subsequently spread. The age group most affected were children between 1 and 4 years who comprised 47.7% of reported cases. Most of the remaining cases were in children aged 5–14 (39.5%) or in infants <1 year of age(9.8%). Only 3.1% of reported cases were in those aged ≥15 years. Seven measles associated deaths were reported during the outbreak in children aged 1–4 years. The age distribution of the subset of cases from Bedelle district confirmed by laboratory diagnosis was similar (Table I), although fewer of the laboratory confirmed cases were in children <5 years of age. Reported measles vaccine coverage was less than 40% in the three districts. Of the 1,603 reported cases, 1440 were in nonvaccinated people and only 163 in those who were vaccinated. Management of the epidemic consisted of supportive therapy (antibiotics, oral rehydration, salt administration, and vitamin A supplement), and health education. Schools in the affected area were closed for approximately 1 month, and 7,823 children aged <5 years in adjacent villages were given measles vaccine.

TABLE I. Age Distribution of Reported and Laboratory–Confirmed Measles Cases

Age group (years)	Reported cases (%)	Confirmed cases (%)
0– 1	156 (9.8)	2 (3.8)
1– 5	762 (47.7)	14 (26.4)
5– 10	393 (24.6)	19 (35.8)
10– 15	238 (14.9)	15 (28.3)
15+	50 (3.1)	3 (5.7)
Total	1,599	53

Laboratory Confirmation

Of 55 cases from the Bedelle district, 53 were confirmed as recent measles by both IgM serology and RT-PCR with the primer set for amplifying a 550-nt region of the H gene. The two cases that were not confirmed were an infant of 7 months and an 18-year-old girl with a clinical diagnosis of varicella. Of the 53 confirmed, 40 (75%) were measles IgM positive in oral fluid samples. The oral fluid measles IgM detection rate increased from 65% at days 2 and 3 to 100% at day 6 (Table II). The two sporadic cases from Addis Ababa were both IgM and RT-PCR positive on oral fluids.

Genetic Characterisation of Measles Virus Strains

Of the 53 RT-PCR positive specimens, 28 chosen at random from the Bedelle outbreak and two from the sporadic cases in Addis Ababa were sequenced, initially over a 450-nt region of the H gene. Identical sequences were obtained in all 28 specimens from the Bedelle outbreak, and two distinct strains were identified from the sporadic cases. Subsequently, the entire H and N genes of these three representative strains were amplified by RT-PCR, and the amplicons were directly sequenced. The three strains were named as MVs/Bedelle.ETH/5.99 (ETH10/99) from a 5-year-old child

TABLE II. Oral Fluid Measles-IgM Detection and Date of Sample Collection in Days After Onset of Symptoms in Laboratory-Confirmed Cases from the Bedelle Outbreak

Oral fluid measles-IgM	Day after onset of symptoms							No. of cases
	2	3	4	5	6	7	8	
Pos	2	10	10	8	3	7	0	40
Neg	1	6	3	2	0	0	1	13
%	67	63	77	80	100	100	—	75

from the Bedelle outbreak and MVs/Addis Ababa.ETH/50.98 (ETH54/98) and MVs/Addis Ababa.ETH/2.99 (ETH55/99) from sporadic cases in Addis Ababa, respectively, in a 10- and in a 2-year-old child (GenBank accession numbers AF280800–280802, 280805–280807).

The entire N gene of two strains, UK140/94 and UK160/94, identified in the UK in 1994 [Jin et al., 1998b] was also sequenced (GenBank accession numbers AF280803 and AF280804) for comparison, and these two strains were renamed as MVi/Manchester.-UNK/30.94 and MVi/Manchester.UNK/32.94 according to the WHO nomenclature [WHO, 1998].

Figure 2 shows the phylogenetic relationships between the three Ethiopian and 33 other measles virus strains including 18 WHO reference strains [WHO, 1998], 10 previously reported African strains [Truong et al., 1999], one suspected Indonesian strain assigned to genotype G2 [de Swart et al., 1999], one Australia strain assigned recently to genotype D7 [Chibo et al., 2000] and three UK strains, UK140/94, UK160/94, and UK226/94 identified in 1994 [Jin et al., 1998b) which are closely related to ETH54/98. The phylogenetic tree in Figure 2 was constructed by analysing the entire H gene sequence (1854-nt). The three Ethiopian (ETH) strains clustered in the D clade but were located on two different branches. Strains ETH10/99 and ETH55/99 were closest to genotype D4, and there were, respectively, 32 (1.7%) and 29 (1.6%) nt differences and 7 (1.1%) and 3 (0.5%) aa differences between these two Ethiopia strains and the D4 reference strain (Montreal.CAN/89). There was at least 2.5% divergence between these two Ethiopia strains and other genotypes of clade D. Strain ETH54/98 showed at least 2.4% divergence to all reference genotypes but more than 98% similarity to the three UK strains, UK140/94, UK160/94, and UK226/94, and together with these three strains, formed a new branch. The degree of divergence would suggest that this group should be considered an additional genotype of clade D.

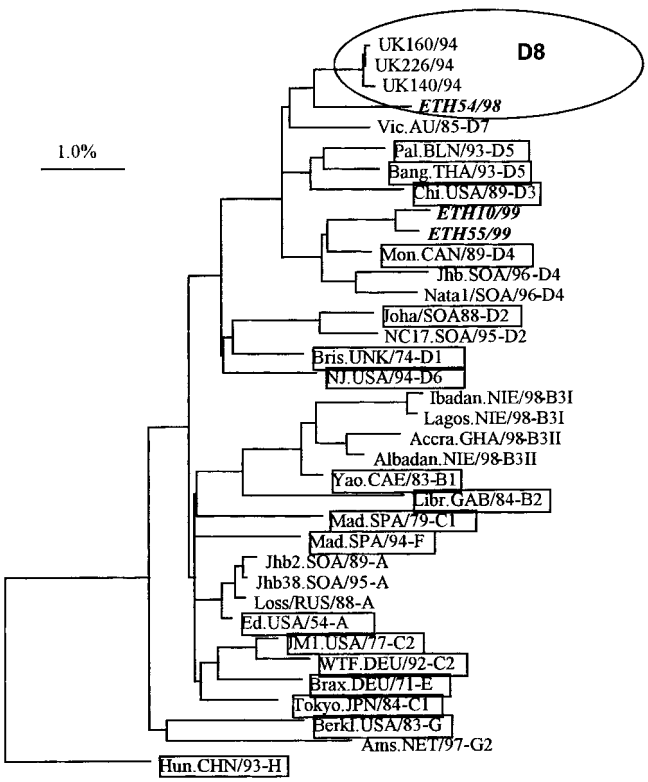

Fig. 2. Genetic relationships between measles strains detected in Ethopia and previously reported genotypes. Unrooted tree diagram was constructed on the basis of the entire H gene (1854 nt) sequence using the clustal method (Megalign programme, DNASTAR). Strains reported in this study are in Italics. WHO reference strains are in boxes and genotypes assigned previously are indicated at the end of the strain designation.

A similar phylogentic tree based on the C-terminus of the N gene was constructed by the neighbour-joining method of the PAUP 4.0 Beta 2 package and subjected to bootstrap analysis (Fig. 3). Strains ETH10/99 and ETH55/99 were close to genotype D4 but strain ETH54/98 appeared on a new branch with the two of the three UK strains mention above (UK140/94 and UK160/94). This confirmed phylogenetic relationships based on analysis of the H gene. On the basis of the entire N gene sequences, there were 34 (2.2%) and 32 (2.0%) nt differences and 8 (1.5%) and 5 (0.9%) aa differences between the Ethiopia strains ETH10/99 and ETH55/99, respectively, and D4 reference strain (Montreal.CAN/89), while there was at least 3% divergence from other reference genotypes. There were 17 (1.1%) nt differences between strain ETH 54/98 and the UK strains and at least 2.7% divergence from other genotypes. This new genotype is therefore proposed as genotype D8 (Figs. 2 and 3) using MVi/Manchester.UNK/30.94 (UK140/94) as the reference strain. This isolate is available at the WHO Measles Strain Bank (Enteric and Respiratory Virus Laboratory, Central Public Health Laboratory, London) and the sequences of entire H and N gene are available at the GenBank.

Amino acid differences between the three Ethopia strains and closely related measles strains are sum-

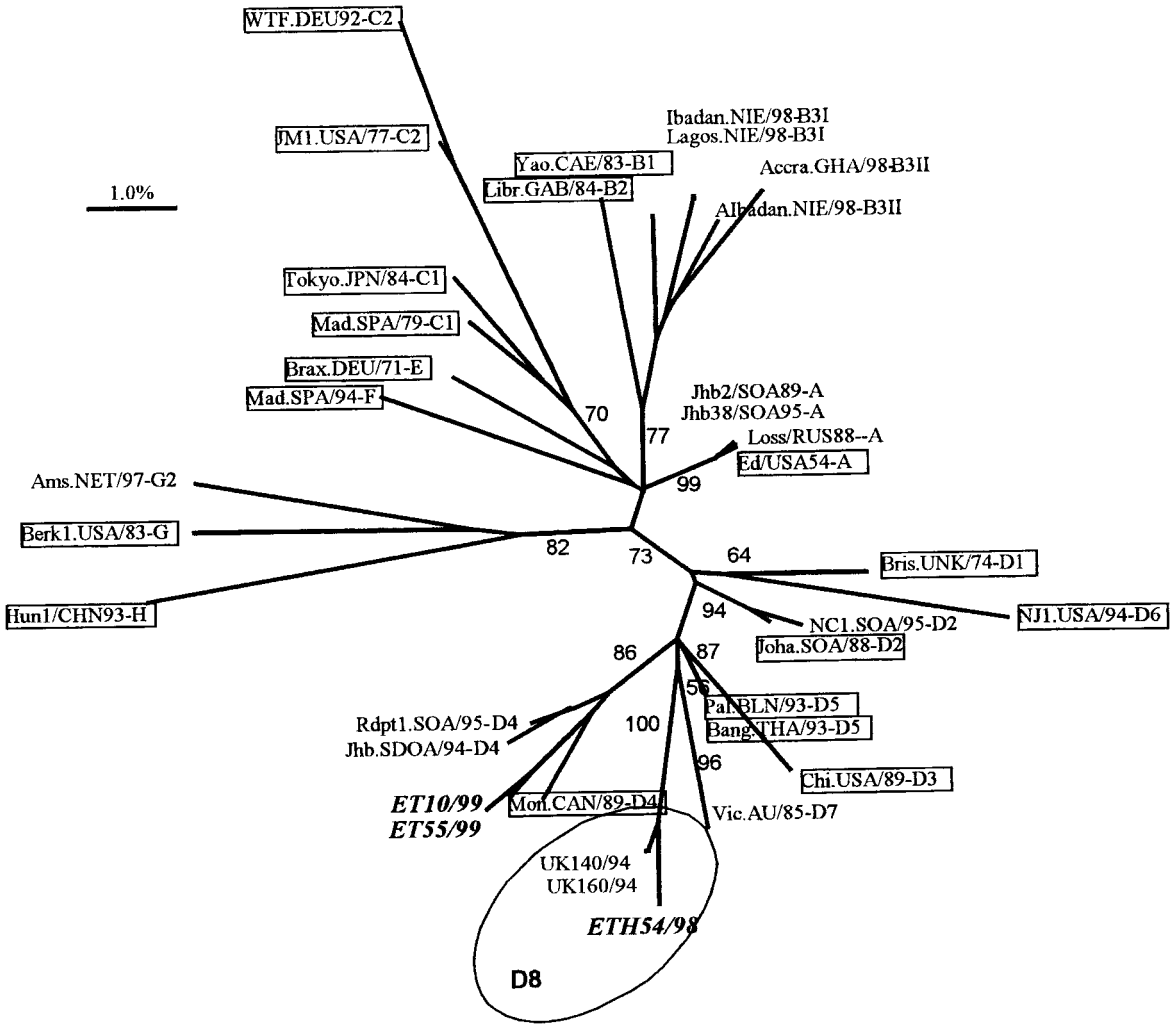

Fig. 3. Genetic relationships between measles strains detected in Ethopia and previously reported genotypes on the basis of the C-terminus of the N gene (456 nt). The phylogenetic tree was drawn by bootstrap analysis (1,000 times) using the neighbour-joining method (PAUP 4.0 Beta 2 package). Measles strains reported in this study are in italics, and the reference strains (in boxes) with the designated WHO measles genotype are indicated at the end of each strain designation.

marised in Table III. Amino acids coded by consensus nt sequences (con) were based on the sequences of strains selected for phylogenetic analysis which covered all measles virus genotypes defined by the WHO [1998]. Compared with the aa sequence of the Mont89/D4 strain, there were seven (ETH10) and three (ETH55) aa differences in the H gene and eight and five aa differences in the N gene. Compared with the aa sequence of the UK140/94 strain, there were 13 aa differences in the H gene of strain ETH54 but no aa differences in the N gene because, although there were 17 nt substitutions, all were synonymous.

DISCUSSION

Factors contributing to the spread of measles in the outbreak included the low vaccination coverage of less than 40% and probably the gathering of people in schools and market places, although this is conjecture. The age groups affected were principally children aged <1–14 years, as observed in England and Wales before the introduction of measles vaccine [Benjamin, 1968] where school aggregation played a key role in seasonal epidemicity [Fine and Clarkson, 1982]. Although the system of notification is far from perfect, leading to gross underreporting, it is of interest that in the 3 years before the outbreak no measles cases had been reported in the Illubabor zone. It seems likely that measles is not endemic in this remote rural part of Ethiopia but introduced periodically from endemic locations such as urban centres. The similarity of the genotype in one sporadic case from Addis Ababa and in the outbreak in Bedelle supports this conjecture.

Although infections with other agents such as rubella, parvovirus B19, human herpes virus-6 (HHV-6) and group A streptococcus may resemble measles clinically, the diagnosis of measles cases in this study by staff at health centres was mostly confirmed where laboratory investigations were done. Of the 55 clinically diagnosed cases investigated in the Bedelle outbreak 53

TABLE III. Amino Acid Differences Between Ethiopia and Closely Related Measles Strains*

			Measles strain				
			D4			D8	
Gene	Amino acid	Con[a]	Mont 89	ETH10	ETH55	UK140	ETH54
H	34	R	...	K	K	...	...
	79	D	...	N	...	...	...
	120	D	...	...	...	N	...
	160	E	...	...	...	...	D
	174	T	A	A	A	A	A
	176	T	A	A	A	A	A
	192[b]	**T**	...	...	...	...	P
	194[b]	**T**	...	...	...	...	L
	196[b]	**G**	...	S	...	...	...
	212	R	...	...	...	...	Q
	247	S	...	...	...	...	P
	252	Y	H	H	H	H	H
	283	L	F	F	F	F	F
	296	L	F	F	F	F	F
	302	G	R	R	R	R	R
	305	S	...	A	A	...	...
	307	T	...	...	...	A	A
	366	A	...	V	...	...	...
	400	A	...	...	...	T	...
	416[b]	**D**[b]	**N**	**N**	**N**	...	**N**
	451	V	...	...	...	M	M
	473	I	V	V	V	...	...
	517	L	...	...	...	...	...
	546	S	G	...	...	...	...
	560	K	...	...	...	R	...
	577	L	...	...	...	P	...
	594	I	...	...	...	...	L
	608	V	...	...	...	A	...
	612	D	...	...	...	E	...
N	61	N	D	...	...	...	...
	137[b]	S	...	**G**	...	...	...
	138[b]	S	...	**I**	...	...	...
	139[b]	S	...	**G**	**G**	...	...
	144[b]	F	S	**S**	**S**	**S**	**S**
	406	I	...	...	...	T	T
	420	L	I	I	I	...	...
	441	K	...	...	...	R	R
	450	S	...	N	...	...	...
	451	Y	...	...	...	N	N
	456	P	S	S	S	S	S
	459[b]	**A**	...	**T**	**T**	**L**	**L**
	470[b]	**G**	**S**	**S**	**S**	**S**	**S**
	482	S	G	G	G	G	G
	514	T	I	I	I	...	...
	521[b]	**R**	**K**	...	...	...	...
	522[b]	**D**	...	**N**	**N**	**N**	**N**

*All amino acid differences between Ethiopia and closely related measles strains are shown.
[a]Differences at important functional sites are bold face.
[b]Amino acid coded by consensus nucleotide sequences in Figs. 1 and 2, which include all MV genotypes defined by the WHO.

were confirmed as recent measles by IgM testing and PCR. Two cases were not confirmed. One was an adult in whom varicella was suspected. The other was a 7-month infant. A frequent cause of measles misdiagnosis in this age group is roseola infantum, primary infection with HHV-6 [Tait et al., 1996]. These results indicate that the WHO case definition is specific in this epidemiological context.

In this study, only 75% of confirmed cases were positive by measles-IgM defection in oral fluid. The low diagnostic yield from oral fluid IgM testing was due to the early collection of samples, which was between 2 and 8 days (mean = 4.4 days) after onset of symptoms. Previous studies have shown that the optimal time for detecting measles IgM in oral fluid is 1–5 weeks after onset of symptoms [Perry et al., 1993]. Conversely, the early collection of samples favoured the detection of measles virus RNA and in this study all cases confirmed by measles-IgM detection in serum were RT-PCR positive on corresponding oral fluids.

Sequence analysis of measles RNA amplified by RT-PCR identified three distinct measles strains. All 28

strains from the Bedelle outbreak were identical and closely related to genotype D4 [WHO, 1998]. One of the strains from sporadic cases in Addis Ababa was also similar to genotype D4 but the ETH54 strain from Addis Ababa was distinct. It was closely related to strains identified in the UK in 1994 (Figs. 2 and 3). While strain ETH54 could be assigned to clade D, it was not identical to any genotype described previously within this clade and might be representative of a genotype not described previously. The occurrence of this new genotype, in addition to the genotypes A, B1, B2, B3, D2, and D4 identified previously in Africa, suggests that genetically distinct measles virus lineages are co-circulating in Africa. The results of this study indicate the need for continued molecular and antigenic surveillance of wild-type measles strains.

Analysis of aa sequence variation in the entire H and N genes of three Ethiopia strains revealed that there were significant changes (Table III) affecting sites recognised currently as having important biological and immunological functions. These sites include the predicted B-cell epitope at aa309–318 of the H gene [Makela et al., 1989; Partidos, et al, 1991] and three antigenic sites at aa122–150, aa457–476, and aa519–525 of the N gene [Buckland et al., 1989; Komase et al., 1990]. Amino acid 416 of the H gene, which is postulated to affect hemagglutination [Saito et al., 1995], had the same change in all three Ethiopia strains compared to the aa coded by the concensus nt sequence (Table III). The nt substitution G to A, which results in the change at aa416, produces an additional potential N-linked glycosylation site which has been found in isolates from Japan [Saito et al., 1995] and the United States (Chi1/89, D3; WHO reference strain). Analysis of the nt sequences of strains ETH10 and ETH55 (detected in 1998/1999], the reference genotype D4 strain (isolated in 1989), strain ETH54 (detected in 1998/1999) and the closely related UK strains (isolated in 1994) showed significant genetic drift within clade D. Furthermore, in previous studies, antigenic reactivity demonstrated by different monoclonal antibodies indicated that current wild-type measles virus genotypes contain strain specific epitopes [Jin et al., 1998b; Komase et al., 1990]. Further studies to attempt isolation of the Ethiopia measles strains in cell culture and to map their antigenic epitopes and genetic evolution are currently in progress.

Effective surveillance is fundamental to the evaluation of measles vaccination programmes and, within this, genotyping plays an important role [Cutts et al., 1999; Rota et al., 1996], particularly in the identification of transmission pathways as measles elimination is approached. Accelerated control programmes, including measles vaccination campaigns, will increase the need for antibody detection and genotyping methods appropriate to developing country settings. Our study demonstrates that oral fluid collection, which is non-invasive and technically simple, can assist in attaining this objective.

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