Cocirculation of Measles Virus Genotype B2 and B3.1 in Central African Republic During the 2000 Measles Epidemic

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Many African countries have begun implementation of national programs to eliminate measles by the year 2015. However, measles continues to be endemic in Africa. This study describes the first molecular epidemiological study of measles virus circulating in Central African Republic. Two hundred and ten blood samples were tested for measles IgM. Sixty-seven urine samples were collected during measles outbreak in Bangui in 2000 and 2004 and used for genotyping studies. Two different methods were used to determine measles virus genotypes; the recently described real-time PCR-based method and the nucleotide sequencing and phylogenetic analysis methods. These tests revealed the cocirculation of two distinct viruses in Bangui. The proposed subgroup of the B3 genotype, B3.1 was found in 14 samples. This virus has been found in other neighboring countries. More surprising, genotype B2 was found in samples from four patients. The first measles genotype B2 viruses were isolated in Gabon in 1984, but have not been detected until recently when they were identified during a measles outbreak in 2003 in South Africa. This suggests that the circulation of measles genotype B2 has continued in Central Africa during the last 20 years. This study provides the baseline for genetic surveillance of measles virus in Central African Republic. Knowledge of currently circulating measles virus genotype in Central African Republic will help in monitoring the success of measles elimination program. J. Med. Virol. 78:964–970, 2006.

KEY WORDS: measles; molecular epidemiology; Central African Republic

INTRODUCTION

Measles is one of the most infectious diseases of childhood. Despite the use of a live attenuated vaccine for more than 40 years, measles still causes nearly 530,000 deaths annually worldwide, half of which occurs in Sub-Saharan Africa [WHO, 2005].

Serologically, measles virus is considered to be monotypic, that is, one serotype, but molecular epidemiological studies have shown that a number of genotypes exist and co-circulate, [Taylor et al., 1991; WHO, 1998, 2001a]. In the present classification, there are eight clades (A–H) subdivided in 23 genotypes, including a new genotype D10 [Muwonge et al., 2005]. The molecular epidemiological approach plays a key role in tracing the origin of viruses and monitoring the efficiency of vaccination programs. In 1998, WHO implemented measles eradication strategies. For this purpose, WHO recommends that virological surveillance be established in every country. Measles laboratory surveillance includes serological confirmation of suspected measles cases and genetic characterization of measles wild-type virus strains during outbreaks.

The Central African Republic has an estimated population of 3.91 million (2004). In Central African Republic, measles is considered one of the six major

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causes of morbidity and mortality among children. A national program of immunization was established by the Central African Republic authorities in 1988, but the measles vaccine coverage is insufficient (54% in 2004). Since 2000, the Ministry of Health and Population in collaboration with the WHO has established an immunization program with the aim of eliminating measles.

In this study, the first molecular characterization of measles viruses from the Central African Republic is described. Two different methods were used to determine the genotype of these measles virus strains. The conventional method based on sequencing and phylogenetic analysis and a new method based on real-time amplification refractory mutation system PCR (RT-ARMS PCR) using SYBR Green fluorescent dye. It was found that measles virus strain belonging to the proposed subgroup B3.1 and also the genotype B2 co-circulated in Bangui in 2000. Comparison of the sequences with earlier African isolates enables a more complete understanding of the epidemiology of measles in Central Africa region.

MATERIALS AND METHODS

Patients and Samples

All samples (blood and urine) except one, were collected from March to August 2000 from patients with clinically diagnosed measles living in Bangui, Central African Republic. One additional sample was obtained in July 2004. Two hundred ten (98%) of the 214 measles cases reported in the Pediatric Unit of the Centre National Hospitalier Universitaire de Bangui, were tested for measles specific IgM and 67 (31%) urine samples for RNA extraction and genotyping.

ELISA

Measles virus-specific serum IgM was determined with the Enzygnost anti-measles virus IgM test (Dade Behring Marburg GmbH).

RT-PCR and PCR

Viral RNA was extracted directly from the urine samples of 67 patients collected from March to August 2000 and one in July 2004, using the Quiagen viral RNA extraction kit (Quiagen, Courtabouef, France) according to the supplier’s protocol. Specific cDNA of the measles virus nucleoprotein (N) gene was synthesized by reverse transcription immediately followed by polymerase chain reaction (PCR) amplification using Superscript One-Step reverse transcription (RT)-PCR with platinum Taq (Invitrogen, Paisley, UK).

The N gene primers MVNPCR2 (nt 975–996, 5’GCTG GTGAGTTATCCACACTTG3’) and MVNPCR4 (nt 1, 701–1,722, 5’GTAGCCGGATTTGTTCTCTGTT C3’) were used to amplify a 747-bp fragment. The PCR cycling program consisted of denaturation for 5 min at 94°C followed by 35 cycles 30 sec at 94°C, 45 sec at 55°C, 45 sec at 72°C with a final extension for 7 min at 72°C. All products were held at 4°C. These products were used for sequencing and RT-ARMS PCR genotyping reactions.

Genotyping by RT-ARMS PCR

A new method was developed recently to genotype measles virus by real-time ARMS PCR [Waku-Kouomou et al., 2006]. Briefly, the multiple alignment of the 3’ region of N gene sequences were studied and genotype-specific nucleotides also called single nucleotide polymorphisms (SNPs) were identified. Pairs of primers each with an SNP at the 3’ end for genotypes A, B2, C2, and subgroup B3.1 were designed. In order to increase the specificity of these primers, mismatches were introduced deliberate near the SNP. The method uses genotype specific primers to amplify individual measles virus genotypes. It allows real-time detection of PCR products and genotype determination by melting curve analyses.

RT-ARMS PCR was carried out using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Langen, Germany). A master mix reaction was prepared and dispensed in 15 μl aliquots into thin-walled micro-Amp optical tubes (Applied Biosystems). Then 5 μl containing 0.26 ng of N gene PCR product were added to each tube. The final reaction mixture contained 400 nM of each primer, 12.5 μl SYBR Green qPCR SuperMix UDG, 0.5 μl Rox reference Dye (Invitrogen) and RNase free water to complete the reaction volume to 20 μl. All reactions were performed in duplicate. Samples were screened with genotype-specific primers for genotype A (WarmsAs, nt 1,574–1,596, 5’GAAGGTACGCTGACCCCTG GaT3’ and WarmsAs2, nt 1,644–1,665, 5’GATTTCGTCAITGTTACA CAT3’), genotype B2 (WarmsB2s nt 1,400–1,422, 5’GATTGGGG GCGAGGAAGATtcA3’ and WarmsB2as, nt 1,500–1,522, 5’GTGTTGCCGGTTGGAAGAT GccTG3’), subgroup B3.1 (WarmsB3.1s, nt 1,417–1,439, 5’GACAGGAGGTCAACAGctC3’ and WarmsB3.1as, nt 1,587–1,608, 5’GGCTTGCACCTAAGCAG Ggc3’), and genotype C2 (WarmsC2s, nt 1,292–1,314, 5’CAGAGATTGCAATGCATCtaG3’ and WarmsC2as, nt 1,450–1,472, 5’GCCCGGTTCCTCTGTAG ACTc3’). Mismatched base pairs are indicated in small letters.

The PCR reaction was carried out as follows: an initial Uracil DNA Glycosylase (UDG) decontamination step at 50°C for 2 min was followed by a hot start denaturation step at 95°C for 10 min. Next, PCR amplification was carried out for 40 cycles at 95°C for 15 sec and 60°C for 1 min. The fluorescence was read during the reaction allowing a continuous monitoring of the amount of PCR product. After amplification, melting curve analysis was performed on the product by heating from 60 to 95°C for 20 min.

The real-time PCR data were analyzed with ABI Prism 7000 SDS v1.1 data analysis software (Applied BioSystems). The melting temperature (Tm) indicated by the derivative melting curves in each samples was

used to identify the measles virus genotype. The samples whose melting curve had the same Tm point with expected genotype-specific Tm were interpreted as belonging to that genotype. Tm determined for genotypes A, B2, C2, and subgroup B3.1 was 83.2, 85.2, 82.4, and 87.3°C, respectively.

Sequencing

PCR product were electrophoresed on a 1.2% agarose gel, then purified using a QIAquick Gel Extraction Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. Purified PCR products were sequenced with the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Langen, Germany). The reaction products were analyzed on an ABI Prism 3100 automatic sequencer (Perkin Elmer, Langen, Germany). Sequence data were analyzed using the Clustal X program version 1.81 [Jeanmougin et al., 1998] for multiple alignments and construction of phylogenetic tree. This was confirmed with 1,000 bootstraps replicates.

RESULTS

The Epidemiological Position of Measles in Central African Republic

In the Central African Republic, the measles vaccination coverage has been decreasing since 1995. From 52% in 1995, it descended to 33% in 2000 and 29% in 2001 (Fig. 1a). During the same period, mortality due to measles cases progressed from 0.52% in 1999 to 13% in 2003 (Fig. 1b). In 2000, 3,373 measles cases were reported in Central African Republic (Fig. 1b). Fortunately, with the immunization program established by the WHO in 2000, the vaccination coverage is now increasing.

Measles Investigation in the Central African Republic

With the exception of one sample that was collected in July 2004, the remaining specimens were collected between March and August 2000. Among the 210 cases investigated, 82 were vaccinated according to their “parent memory” (Table I). During this investigation, 26 patients died of measles in Bangui’s Pediatric Unit.

Blood samples were examined for measles specific IgM (Table I). Of the 210 cases of clinically diagnosed measles, measles specific antibody was detected in 109 (52%). The age distribution of the laboratory confirmed cases showed that they were mainly in the 6–12 month age group. They represented those with the lower vaccination coverage.

Table I: Age Distribution of Clinically Diagnosed and ELISA-Confirmed Measles Cases

<table>
<thead>
<tr>
<th>Age group (month)</th>
<th>Clinically diagnosed cases</th>
<th>ELISA-confirmed (IgM) cases (%)</th>
<th>Vaccinated cases (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6</td>
<td>6</td>
<td>5 (83)</td>
<td>1 (16)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>6–12</td>
<td>31</td>
<td>28 (90)</td>
<td>4 (13)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>12–60</td>
<td>78</td>
<td>35 (45)</td>
<td>31 (40)</td>
<td>12 (15)</td>
</tr>
<tr>
<td>60+</td>
<td>96</td>
<td>41 (43)</td>
<td>46 (48)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Total</td>
<td>210</td>
<td>109 (52)</td>
<td>82 (39)</td>
<td>26 (12)</td>
</tr>
</tbody>
</table>

Vaccination and mortality rates are shown. ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M.

correctly the 14 samples amplified by genotype B3.1-specific primers, as belonging to subgroup B3.1. In fact, their Tm value of 87.3 °C corresponds to the expected Tm for subgroup B3.1 (Fig. 2, Table II). While analyzing the melting curve profile of the four other samples amplified by genotype B2-specific primers, their Tm value of 85.9 °C did not correspond to the expected Tm for genotype B2, which is 85.2 °C. However, they are very close to each other as the shift is only 0.7 °C. Based on the fact that these primers were designed to amplify only genotype B2 samples, as each primer carries a specific-genotype B2 SNP, it was presumed that these samples belonged to genotype B2 (Fig. 2, Table II).

To verify the accuracy of the RT-ARMS PCR results, the RT-PCR product was sequenced. The 3' region of the N gene was sequenced and analyzed for the 18 samples. Comparison of Central African Republic viruses with the reference sequences defined by the WHO [WHO, 2001a], clearly showed that they belonged to two distinct genotypes. Fourteen belonged to subgroup B3.1 and the other four belonged to genotype B2 (Fig. 3). These results were consistent with the results of RT-ARMS method. The genotype B2 strains used to establish the RT-ARMS genotyping method were isolated in Gabon in 1984 and were the only genotype B2 sequences available on the Web at that time. Sequence comparison with the Central African Republic 2000 samples showed two base pair changes compared with the amplicon sequence. We presume that these changes are responsible for the Tm shift.

### Comparison With Other African Measles B3.1 Viruses

The Central African Republic B3.1 virus sequences were relatively heterogeneous with a maximum divergence of 1.7%. When compared with earlier African B3.1 viruses [Hanses et al., 1999; El Mubarak et al., 2002; Kouomou et al., 2002; Mulders et al., 2003], sequences divergence between the viruses varied from 0.2 to 1.9%. The results also showed that all except two (Mvs/Bangui.CAR/31.04 and Mvs/Bangui.CAR/28.00/3) of the viruses from Central African Republic shared three specific silent mutations; C1224A, A1246G, A1516G [nucleotide numbering according to Cattaneo et al., 1989]. The two other strains sequences were more related to Cameroonian and Nigerian strain [Hanses et al., 1999; Kouomou et al., 2002]. This suggests that there were at least two different lineages of B3.1 viruses circulating during that outbreak.

### Comparison With Other B2 Measles Viruses

In contrast to the B3.1 viruses, the Central African Republic B2 virus sequences were, more homogeneous.
as the four sequences were identical. These viruses were compared with the B2 viruses isolated in Gabon in 1984 (Genbank accession numbers DQ267523, UO1994) [Taylor et al., 1991; Rota et al., 1994] and in South Africa in 2002 and Angola in 2003 (Genbank accession numbers AY994533 and AY994534) [Smit et al., 2005]. The results showed a sequence divergence of 1.3 and 1.7%, respectively. Interestingly, the Central African Republic viruses shared four specific mutations; T1201C, T1204C, A1354G, G1438A. They also shared two other mutations (A1270G and T1349C) with the Angolan and South African strains. All of the above
mutations were silent except for the change at 1,349 leading to Y350H in predicted amino acid sequence.

DISCUSSION

This is the first report of the genetic characterization of measles viruses circulating in the Central African Republic. Despite the willingness of the Central African Republic and WHO authorities to increase measles vaccination coverage, this is still very low and reached only 54% in 2004. This is explained in part by numerous civil conflicts which have occurred during the past years, disrupting the effective delivery of health services and negatively affecting vaccination coverage rates.

All the isolates described in this study except one were collected in 2000, in the Pediatric Unit of the Centre National Hospitalier Universitaire de Bangui, during a measles outbreak. The present investigation reveals a very high mortality rate (12%) among children suffering from measles compared to the estimation of case fatality ranging from 3 to 6% in developing countries [WHO, 2001]. In fact, many of the patients presented signs of measles complications on their arrival at the hospital. This highlights the fact that people are not well informed about the disease. The highest mortality rate (67%) was observed among children in the 0- to 6-month group, indicating an absence of maternal antibody.

In this study, 17 urine samples were examined from patients between March and July 2000 and one in 2004, in Bangui. The results showed that these viruses formed a heterogeneous group with up to 5.7% sequence divergence in the 3' region of N gene. Phylogenetic analyses and RT-ARMS genotyping methods showed that they belong to two different genotypes; B3.1 and B2, indicating that both viruses co-circulated in Bangui during this short period. With the RT-ARMS method, it was observed that genotype B2 samples presented a Tm shift (+0.7°C) from the specific Tm value previously defined for measles genotype B2. Further sequences analyses showed that the Tm shift observed probably resulted from nucleotides variations in the amplicon sequence. Genotype specific Tm shift have been observed in other melting curve genotyping studies [Anderson et al., 2003; Payungporn et al., 2004; Yeh et al., 2004]. However, in most cases, such minor intra-genotype Tm shift did not affect the genotyping determination when compared to the significant Tm differences with other genotypes.

Measles viruses belonging to the proposed subtype B3.1 have been reported in neighboring countries and are known to be endemic in Central Africa [Hanses et al., 1999; El Mubarak et al., 2002; Kouomou et al., 2002; Mulders et al., 2003]. They have also been exported to Europe [Mosquera et al., 2005] and have been found recently in northern Africa [Djebbi et al., 2005].

Four samples examined in this study belonged to genotype B2. The first measles genotype B2 was isolated in Gabon in 1984 [Taylor et al., 1991; Rota et al., 1994] and B2 has been recently reported in South Africa in 2002 and in Angola in 2003 [Smit et al., 2005]. The resurgence of genotype B2 in Central Africa suggested that this genotype has probably continued to circulate during the last two decades, but it is still restricted to sub-Saharan Africa. Interestingly, sequence analyses show that they can be differentiated from Gabon 1984 B2 strains as they have six different nucleotides in the 3' region of the N gene. They also differ from the B2 strains recently isolated in South Africa and in Angola. These changes are probably the result of mutations occurring in Gabon's 1984 strain during the last 20 years.

The cocirculation of measles virus of different lineages in the same country has been previously observed [Hanses et al., 1999; Truong et al., 2001; Kubo et al., 2003]. As this is the first study in Central African Republic, it is not known if these viruses are autochthon or if they have been imported from neighboring countries. The endemic circulation of several viruses is characteristic of communities with low vaccination rates. This may be the case in Central African Republic where the vaccination coverage decreased from 52% in 1995 to 29% in 2001, leading to an increasing number of susceptible persons. The present studies show that measles genotype B2 is active in Central African Republic where it co-circulated with B3.1 virus. This phenomenon is being sustained by a large reservoir of susceptible individuals. With an increasing number of countries establishing the genetic characterization of measles in Africa, the knowledge of geographical distribution of genotypes is continuing to grow. Molecular epidemiology studies can be improved by using the RT-ARMS method which provides a rapid and effective way for measles virus genotyping. Continuous virological surveillance in African countries may provide a clear interpretation of the reappearance of measles virus genotype B2 in Central Africa.

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