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Detection of Measles Virus RNA in Urine Specimens from Vaccine Recipients

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Analysis of urine specimens by using reverse transcriptase-PCR was evaluated as a rapid assay to identify individuals infected with measles virus. For the study, daily urine samples were obtained from either 15-month-old children or young adults following measles immunization. Overall, measles virus RNA was detected in 10 of 12 children during the 2-week sampling period. In some cases, measles virus RNA was detected as early as 1 day or as late as 14 days after vaccination. Measles virus RNA was also detected in the urine samples from all four of the young adults between 1 and 13 days after vaccination. This assay will enable continued studies of the shedding and transmission of measles virus and, it is hoped, will provide a rapid means to identify measles infection, especially in mild or asymptomatic cases.

Despite the existence of an effective vaccine, measles virus continues to cause sporadic outbreaks and epidemics of disease in the United States and throughout the world. Most recent outbreaks have involved either children who were too young to be vaccinated or older children and teenagers (5 to 19 years), most of whom had been previously vaccinated (3, 8). Because of the sporadic nature of outbreaks in populations with high rates of vaccination, the altered presentation of clinical signs that occurs in "mild measles" infections (1, 11, 20), and the presence of other exanthem-causing infections, effective public health measures to control measles outbreaks are more dependent on laboratory confirmation of infection than on diagnosis based on clinical presentation. Currently available diagnostic techniques, which include virus isolation, viral antigen detection, and serologic antibody studies, are very sensitive and specific. However, these techniques are labor intensive, require specimen collection by medically trained personnel, and would be inappropriate for screening large numbers of individuals.

The detection of measles virus RNA in urine by using reverse transcriptase-PCR (RT-PCR) would be a potentially rapid means of detecting measles infections with a clinical specimen which is more readily and conveniently accessible than serum or nasopharyngeal aspirates. Collection of urine specimens could be done in the absence of medical professionals, and on-site specimen-processing requirements are minimal. Measles virus can be isolated from urine specimens from infected individuals for as long as 10 days after the onset of the rash (16, 28), and measles antigen has been detected by immunofluorescence in urine samples from asymptomatic case contacts (5).

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Since urine specimens from naturally infected individuals were unavailable at the time this study was conducted, the RT-PCR assay was evaluated by using specimens obtained from recently vaccinated individuals. In all cases, RNA was extracted from urinary sediment by the guanidinium acid-phenol method (9) and resuspended in 25 μl of RNase-free water.

For the measles virus-specific RT-PCR, a nested set of primers that hybridized to the nucleoprotein (N) gene was used (MV41, CAT TAC ATC AGG ATC CGG; and MV42, GTA TTG GTC CGC CTC ATC). The internal primers (MV43, digoxigenin [DIG] -GA GCC ATC AGA GGA ATC A; and MV44, DIG-CA TGT TGG TAC CTC TTG A) were 5′ labeled with DIG. The target sequences for these primers are located between bases 57 and 389 of the coding region of the N gene, and these sequences are conserved among the N genes of all wild-type measles viruses examined thus far (24). DIG-5′-labeled primers that amplified beta-actin mRNA (BA4 and BA1) were used as controls for RNA extraction.

Before the RT reaction, the RNA was heated to 95°C for 90 s and then placed on ice. The RT reaction mixture contained 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 5 mM dithiothreitol, 1 mM each deoxynucleoside triphosphate (dNTP), 10 μM each forward and reverse primer (MV41 and MV42 or BA1 and BA4), 24 U of avian myeloblastosis virus RT, and 40 U of human placental RNase inhibitor. The reaction mixture was incubated at 42°C for 45 min and then at 95°C for 5 min.

For PCR, 5 μl of the cDNA sample was added to a 45-μl PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dNTP, 5 μM each primer (MV43 and MV44 or BA1 and BA4) and 5 U of *Taq* DNA polymerase. PCR conditions were as follows: 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. After 39 cycles, 20 μl of each sample was analyzed by electrophoresis on a 1.5% agarose gel. DNA was visualized by ethidium bromide staining and UV illumination. Immunochemiluminescence detection of PCR products that were not visible after ethidium bromide staining was performed as described previously (10).

In all RT-PCR assays, samples containing water were used as contamination controls. Positive control RNA was extracted from Vero cells that had been infected with measles virus, and negative control RNA was extracted from uninfected Vero cells or from urine specimens donated by laboratory personnel who had not recently been vaccinated. For measurement of the

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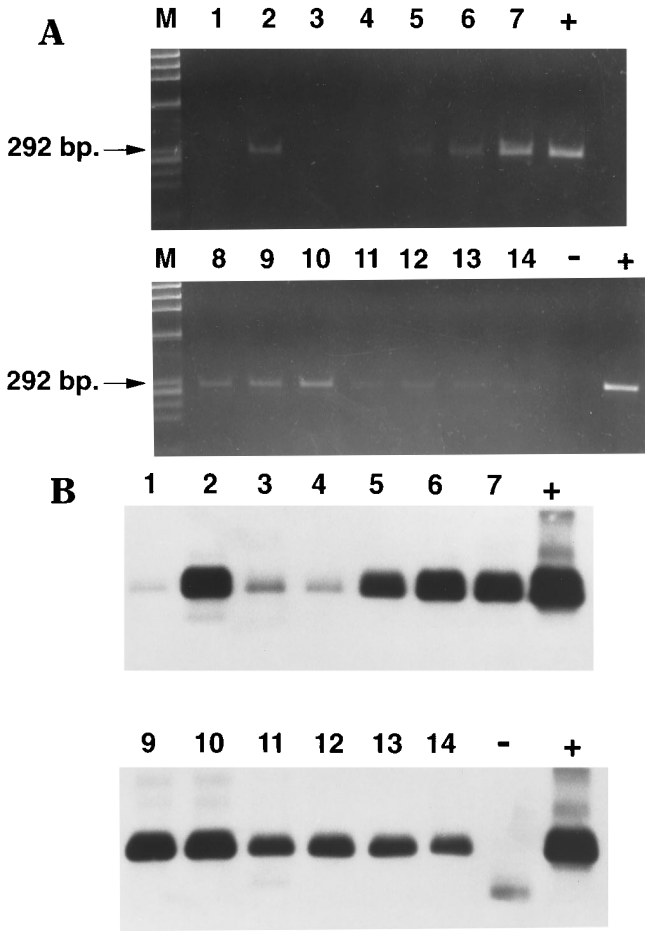

FIG. 1. RT-PCR analysis of urine samples from a single vaccinated child. (A) Agarose gel electrophoretic analysis of PCR products after ethidium bromide staining. Lane numbers indicate the day postvaccination for each sample. Positive (+) and negative (−) controls and molecular size markers (M) are also shown. (B) Chemiluminescence detection of PCR products from the RT-PCRs shown in panel A (the sample from day 8 is missing).

sensitivity of the RT-PCR assay, measles virus N gene RNA was synthesized in vitro from a plasmid template by using T7 RNA polymerase (25).

The product generated by the RT-PCR amplification of measles virus RNA was a 292-bp DNA fragment that was end labeled with DIG during the reaction (Fig. 1). The addition of DIG to the second set of PCR primers increased the sensitivity of detection of the PCR product by approximately 100-fold. As little as 1.5 fg of in vitro-synthesized measles virus N gene RNA could be detected, an amount that represents approximately 10^4 RNA molecules. The number of N gene mRNA molecules in a single infected cell has been estimated to be 1,000 to 10,000 (7). Therefore, the RT-PCR assay was able to detect as few as 1 to 10 infected cells.

First-voided morning urine samples were collected daily from 12 children (age 15 months) over a 14-day period following routine initial measles-mumps-rubella vaccination. The time between collection and sample processing varied from 24 to 72 h. Many of the samples were highly contaminated with bacteria upon arrival in the laboratory, and the volume of urine obtained varied between 5 and 50 ml per specimen. A total of 144 specimens were received from the 12 children over the 14-day study period (there were 24 missing specimens).

TABLE 1. Detection of measles virus RNA in urine samples from recently vaccinated children (n = 12) by RT-PCR

Actin mRNA	No. of samples with measles virus RNA:		
	Present	Absent	Total
Present	48	21	69
Absent	8	67	75
Total	56	88	144

The quality of the RNA extracted from all samples was assessed by using exon-specific primers to amplify a 300-bp region of beta-actin mRNA. The RNA extracted from the urine samples appeared to be free of DNA contamination, since the 300-bp actin PCR product was obtained after DNase, but not RNase, treatment of the sample and there was no evidence for a higher-molecular-weight PCR product that would have been produced by amplification of the beta-actin gene. No actin mRNA was amplified from 57 (52%) of 144 samples, indicating that extensive RNA breakdown had occurred during storage, shipment, or RNA extraction (Table 1).

Measles virus RNA was detected in 48 (70%) of 69 actin-positive samples and in 8 (11%) of 75 actin-negative samples by using either the ethidium bromide or chemiluminescence detection method (Table 1). The detection of measles virus RNA in samples that were negative for actin mRNA could be attributed to increased sensitivity of the measles virus PCR or, more likely, to increased stability of measles virus RNA, which would be associated with nucleocapsid structures. Overall, measles virus RNA was detected in 56 (39%) of 144 samples. Sequence analysis of several of the PCR products confirmed that the appropriate region of the N gene of the measles vaccine strain, Moraten (Attenuvax; Merck, Sharp and Dohme, West Point, Pa.), was being amplified (23). Urine samples donated by laboratory staff were processed in parallel to the samples obtained from the vaccinated children. No measles virus RNA was detected in any of these control samples (data not shown).

In some cases, measles virus RNA was detectable as early as 1 day after vaccination. In four samples, RNA was detected as late as 14 days after vaccination. In Fig. 1, which shows the results from one individual, the PCR product is visible by ethidium bromide staining for 10 of the samples, but all of the samples are positive with the chemiluminescence detection method.

The number of measles virus-positive specimens remained relatively constant during the 14-day sampling interval, with between 1 and 6 of the 12 specimens positive for measles virus RNA on any day (Fig. 2). Overall, measles virus RNA was detected in at least one specimen from 10 (83%) of 12 of the children. Of the 27 samples from the two children in whom measles virus RNA was not detected, only 1 sample was positive for actin mRNA. This extensive RNA degradation was probably due to poor specimen handling at the collection site. For this study, the average numbers of actin-positive samples and measles virus-positive samples were 5.1 and 4.6 per child, respectively.

Urine specimens were also obtained from four healthy young adults (ages 21 to 32 years) for 14 days after they received a booster dose of measles-mumps-rubella vaccine. These samples were of better quality than those obtained from the young children, since larger volumes were obtained and the times between collection, refrigeration, and RNA extraction were shorter. In these cases, RNA was extracted from a max-

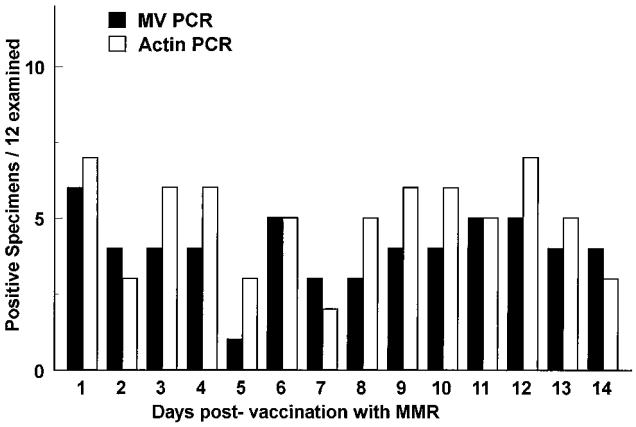

FIG. 2. Time course of detection of measles virus RNA in urine specimens from vaccinated children. Bar heights indicate numbers (total = 12) of measles virus (MV)-positive and actin-positive samples on each day of sampling. MMR, measles-mumps-rubella vaccine.

imum volume of 100 ml of urine; 81% of these samples were positive for actin mRNA. While measles virus RNA was detected in all four individuals (Table 2), it was detected in fewer of the samples and in samples from only two of the individuals after day 2. This suggests that preexisting immunity may have reduced the extent of replication or shedding of the vaccine virus.

During acute infection, measles virus is routinely isolated from the urine for as many as 10 days after the onset of the rash (16, 28). Viral antigen has been detected in multinucleate, giant cells found in urinary sediment by using immunofluorescence (19) before or after cell culture amplification (22). Detailed microscopic and immunofluorescence studies have shown that these antigen-bearing cells are exfoliative cells from proximal renal tubules, collecting tubules, epithelial cells of Bowman's capsule, and the transitional epithelium of the renal pelvis, ureter, and urinary bladder (6, 18, 27), suggesting that the urinary tract is infected during measles infection. Other morbilliviruses, such as canine distemper virus and phocine distemper virus, also infect epithelial cells in the urinary tract (2, 4, 14).

Measles virus antigen has been detected in the urinary sediments of vaccinated individuals by immunofluorescence (19) or, more recently, by RT-PCR (26a). In the study by Llanes-Rodas and Liu (19) in 1966, the urine samples were obtained during a measles vaccine trial. In that case, the test vaccine was an earlier passage of the Edmonston virus (12) that had greater reactogenicity than the more attenuated vaccine, Moraten (17). In our study, individuals received the Moraten strain of measles vaccine as measles-mumps-rubella vaccine.

TABLE 2. Detection of measles virus RNA in urine samples from recently vaccinated young adults[a]

Patient no.	Age (yr)	No. of days[b]	Days measles virus positive[c]
1	21	15	1, 2
2	24	13	1, 2
3	26	16	1, 2, 3, 4, 5, 6, 10, 13
4	32	14	9, 11

[a] All subjects were positive for measles virus immunoglobulin G as determined by enzyme immunoassay.
[b] Number of days after vaccination that specimens were obtained.
[c] Days on which measles virus RNA was detected by RT-PCR.

Measles virus RNA was detected by RT-PCR in the urine specimens from several of the vaccinated children as late as 14 days after vaccination. Because our research protocol was limited to only 14 days of specimen collection, we were unable to determine the upper limit for the duration of viral RNA in urine. In the previous study by Llanes-Rodas and Liu (19), measles virus antigen was detected in urine as late as 16 days after vaccination.

The finding that several of the urine samples were positive for measles virus RNA as early as 1 day after vaccination was surprising. In the previous study (19), none of the urine specimens were positive by immunofluorescence before day 4. Since a single cycle of viral replication would be expected to take 17 to 24 h, it is unlikely that the RT-PCR detected the progeny of virus replicating in the urinary tract. Rather, this observation suggests that shortly after vaccination the input virus or viral antigen, in the form of nucleocapsids, is deposited directly into the bladder via interstitial fluid. This finding also demonstrates the increased sensitivity of RT-PCR compared with the immunofluorescence techniques that were used in earlier studies (18). Unfortunately, most of the specimens were of such poor quality that cytological studies or reisolation of vaccine virus was not attempted. In the previous study (19), attempts to isolate vaccine virus on cell culture were unsuccessful.

The changing epidemiology of measles, in the form of mild measles cases in previously vaccinated individuals (1, 11, 20), suggests that more asymptomatic or subclinical cases might be occurring. The frequency of such infections, which would not meet the standard case definition of the Centers for Disease Control and Prevention, is not known. Also, it is not known whether individuals who do not display the full range of clinical signs characteristic of measles infection are capable of transmitting the virus to other susceptible individuals. In one previous study, urine samples from 5 of 12 measles case contacts were positive for measles virus antigen even though only 1 of these 5 contacts developed clinical signs (5).

In general, RT-PCR has proven to be a rapid and sensitive method to detect measles virus RNA in a variety of clinical specimens (13, 15, 21, 24, 26, 28). Successful RT-PCR amplification of measles virus RNA from urine samples now allows the detection of measles virus RNA from a specimen that can be obtained from a large number of individuals by noninvasive means. We plan to use this assay to define further the extent of asymptomatic or mild infection in case contacts during an outbreak, to determine the role that these cases play in the transmission of measles, and to measure the shedding patterns of vaccine recipients. In future surveys, more care will need to be taken to obtain and process the specimen in a manner that minimizes RNA degradation.

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