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Middle East respiratory syndrome coronavirus not detected in children hospitalized with acute respiratory illness in Amman, Jordan, March 2010 to September 2012

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Abstract

Hospitalized children < 2 years of age in Amman, Jordan, admitted for fever and/or respiratory symptoms, were tested for Middle East respiratory syndrome coronavirus (MERS-CoV): MERS-CoV by real-time RT-PCR (rRT-PCR). This was a prospective year-round viral surveillance study in children <2 years of age admitted with acute respiratory symptoms and/or fever from March 2010 to September 2012 and enrolled from a government-run hospital, Al-Bashir in Amman, Jordan. Clinical and demographic data, including antibiotic use, were collected. Combined nasal/throat swabs were collected, aliquoted, and frozen at −80°C. Specimen aliquots were shipped to Vanderbilt University and the Centers for Disease Control and Prevention (CDC), and tested by rRT-PCR for MERS-CoV. Of the 2433 subjects enrolled from 16 March 2010 to 10 September 2012, 2427 subjects had viral testing and clinical data. Of 1898 specimens prospectively tested for other viruses between 16 March 2010 and 18 March 2012, 474 samples did not have other common respiratory viruses detected. These samples were tested at CDC for MERS-CoV and all were negative by rRT-PCR for MERS-CoV. Of the remaining 531 samples, collected from 19 March 2012 to 10 September 2012 and tested at Vanderbilt, none were positive for MERS-CoV. Our negative findings from a large sample of young Jordanian children hospitalized with fever and/or respiratory symptoms suggest that MERS-CoV was not widely circulating in Amman,

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Dr. Williams reports personal fees from Quidel, outside the submitted work.

Jordan, during the 30-month period of prospective, active surveillance occurring before and after the first documented MERS-CoV outbreak in the Middle East region.

Keywords

Coronavirus; MERS-CoV; novel; surveillance; virus

Introduction

The recent emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) [1,2], first discovered in September 2012, has led to enhanced surveillance efforts throughout the world to detect new cases in a timely manner and to better understand the transmission and pathogenesis of the virus. Coronaviruses are a diverse group of positive-strand RNA viruses that commonly infect the respiratory and gastrointestinal tracts of mammals and birds. Human coronaviruses are most commonly associated with mild respiratory illnesses, but can cause severe illness, such as severe acute respiratory syndrome (SARS) coronavirus. Genetic sequencing data indicate that the MERS-CoV is a betacoronavirus closely related to two bat coronaviruses (HKU4 and HKU5), but distinct from other human coronaviruses, including SARS-CoV [1,3].

As of November 11, 2013, World Health Organization (WHO) has been informed of a total of 151 laboratory-confirmed cases of infection with MERS-CoV, including 64 deaths [4]. The cases have originated from the following countries Middle Eastern countries to date: Jordan, Qatar, Saudi Arabia and the United Arab Emirates (UAE). France, Germany, Spain, Italy, Tunisia and the United Kingdom have also reported laboratory-confirmed cases [2,4, 5]. The first known MERS-CoV infections were detected retrospectively as occurring in April 2012 in Jordan [6]. Based on confirmed cases to date, the clinical syndrome consists of febrile illness and pneumonia, as well as renal failure in some patients. The majority of reported cases have been in adults and 64 of 151 cases have been fatal (42%) [4]. Some cases have occurred in clusters. One cluster involved two cases, both fatal, occurring near Amman, Jordan (Zarga, 20 km in distance), in April 2012 [6]. Stored samples from these two cases (one hospital worker and one student) tested positive for MERS-CoV retrospectively, and this cluster was temporally associated with cases of illness among hospital workers. Several other clusters suggested human transmission [5,7,8]; although the risk of sustained transmission is unknown [2,5].

The epidemiology of MERS-CoV, including its potential distribution among paediatric cases of severe acute respiratory illness (ARI), has not been characterized. A critical question is whether the cases identified thus far are the only MERS-CoV infections or whether the virus has circulated more widely undetected. This is of interest in light of the epidemiology of SARS-CoV, where infections in children < 12 years of age were generally milder, with no mortality and few hospitalizations [9]. To determine whether MERS-CoV was present among young paediatric ARI cases before, during and after the occurrence of the Zarga, Jordan MERS-CoV cluster, we tested upper respiratory tract specimens collected from a

prospective study of paediatric ARI hospitalizations in Amman, Jordan, from March 2010 to September 2012.

Methods

Study design

A prospective year-round viral surveillance study in children <2 years admitted with respiratory symptoms and/or fever commenced on 16 March 2010 at Al Basheer Hospital, the main government-run hospital and referral centre in Amman, Jordan, with 240 paediatric hospital beds and catchment of approximately one million persons (50% of the population). Subjects were enrolled 5 days per week (Sunday–Thursday) for approximately 30 months from March 2010 to September 2012. After consent was obtained, the child's clinical symptoms before admission to the hospital and demographic information were collected by parental interview. Medical records were reviewed to determine the hospital course and clinical outcomes. Institutional Review Board approval was obtained from Vanderbilt University, Jordan University and the Jordanian Ministry of Health.

Specimen collection

Nasal and throat swabs were collected and combined in transport medium (M4RT®, Remel, Lenexa, KS, USA), aliquoted into MagMAX™ Lysis/Binding Solution Concentrate (Life Technologies, Grand Island, NY, USA), snap frozen, and stored at −80°C. Original and lysis buffer aliquots were shipped to Vanderbilt University on dry ice for testing by real-time RT-PCR (rRT-PCR) for respiratory syncytial virus, human metapneumovirus, rhinovirus, influenza A and B, and parainfluenza virus one, two and three [10]. Samples tested by Vanderbilt that had no viruses detected were sent to the CDC for MERS-CoV testing and samples not previously tested for any respiratory viruses were tested for MERS-CoV at Vanderbilt.

Laboratory methods

At Vanderbilt. PCR testing—Nucleic acid extraction and real-time RT-PCR (rRT-PCR) testing—RNA was extracted from thawed specimens according to the manufacturer's protocols for the MagMAX-96™ Viral RNA Isolation Kit (Life Technologies). Primers and probes were synthesized by a commercial supplier (Eurofins MWG Operon, Huntsville, AL, USA) based on previously described sequences [11]. rRT-PCR assays were designed to target the upE and ORF1b regions of the MERS-CoV genome. As a positive control, the target sequence of the upE assay and flanking nucleotides (*c.* 400 bp total) were synthesized by a commercial source (Genscript, Piscataway, NJ, USA) and cloned into plasmid pUC57. One-step rRT-PCR reactions were prepared using the AgPath-ID One-Step RT-PCR kit (Life Technologies) according to the manufacturer's instructions with 400 nM forward and reverse primers and 200 nM probe. Cycling parameters were 50°C × 30 min, 95°C × 10 min and 45 cycles of 95°C × 15 s and 58°C × 30 s, with fluorescence data collected during the 60°C annealing/extension step. Specimens were tested first by the upE assay using 5 μL of RNA. Potential positives were then retested using the ORF1b assay and the upE assay was repeated, both with 10 μL of RNA.

Culture—Cells and viruses—Vero and LLC-MK2 cells were maintained in OptiMEM® (Life Technologies) media supplemented with 2% fetal calf serum, gentamicin, amphotericin B, glutamine and tylosin. All virus work was performed in a biosafety level (BSL) three laboratory following IBC-approved BSL-3 procedures.

Virus recovery—Vero and LLC-MK2 cells were plated in 25-cm^2 flasks. The next day, media was removed and then 1 ml was added back. Frozen specimen aliquots were thawed on ice and 75–100 μL of specimen or PBS (mock) were added to the flasks. Flasks were maintained at 37°C, 5% CO_2 for 30 min, with rocking every 10 min, and then 2 mL of Opti-MEM was added to each flask and flasks were returned to the incubator. Flasks were examined for cytopathic effect (CPE) every 24–48 h.

At CDC. PCR testing—Briefly, total nucleic acids (TNAs) were extracted from 200 μL of the swab specimens using the NucliSENS® easyMAG® (bioMérieux, Durham, NC, USA). All specimens were tested by three discrete rRT-PCR assays: the upE assay as described above and two in-house developed assays targeting the MERS-CoV nucleocapsid gene. All assays were performed in 25 μL final reaction volumes containing 5 μL of sample TNA extract using the Invitrogen SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Life Technologies) on an Applied Biosystems® 7500 Fast Dx Real-Time PCR instrument (Life Technologies). Positive test results were assigned to samples generating well-defined fluorescent curves that crossed the threshold within 45 cycles for any assay and were further investigated. Positive (MERS-CoV RNA transcript template) and negative (nuclease-free water) controls were included in all runs to monitor assay performance. All samples were also tested by rRT-PCR for the presence of the human ribonuclease P gene as a control for specimen quality.

Results

Of the 2433 subjects enrolled from 16 March 2010 to 10 September 2012, 2427 specimens were available for PCR testing for MERS-CoV and had clinical data available. The median age was 3.5 months, 60% were male, and 10% had an underlying medical condition, 30% required oxygen, 11% had the diagnosis of pneumonia and 7% were admitted to the ICU. In addition, 2% attended day care and 76% were exposed to smokers. The majority (97%) of the fathers were employed, and 39.6%, 44.8% and 10% of the mothers finished primary education, secondary education and college, respectively. The majority of children lived in Amman; however, some children lived in the surrounding cities, including Zarqa (34 children, 1.4%). Table 1 includes demographic data, diagnoses and outcome data for the entire group and by testing sites.

Of 1898 specimens prospectively tested for other viruses during 2-year surveillance period between 16 March 2010 and 18 March 2012, 474 samples did not have other common respiratory viruses detected. These samples were tested at CDC for MERS-CoV and none were found to be positive by rRT-PCR for MERS-CoV. The remaining 531 samples collected from 19 March 2012 to 10 September 2012 were tested at Vanderbilt using the MERS-CoV upE assay with 5 μL of RNA extract per reaction. Four of the samples gave positive reactions with high cycle threshold (C_t) values (*c.* 38). These specimens were

retested using the upE and Orf1b rRT-PCR assays with 10 μL of RNA per reaction and all were negative. Finally, these specimens were retested using the CDC MERS-CoV nucleocapsid primers and probes provided to Vanderbilt and were also negative. These four specimens were then inoculated into Vero and LLC-MK2 cells. Three of the samples showed evidence of bacterial or fungal contamination within 24–48 h following inoculation and were discarded and not recultured due to lack of an adequate amount of samples. Cells inoculated with the fourth sample were monitored for 7 days and then passaged into new cultures and followed for an additional 10 days. No CPE was detected.

Comparisons among the three groups were made using the Kruskal–Wallis test or Pearson chi-square test for continuous variables and categorical variables, respectively.

Discussion

Despite the close proximity of the first documented MERS-CoV outbreak in Zarga in April 2012, we did not detect MERS-CoV among children < 2 years of age who were hospitalized with ARI during that same time period in Amman, Jordan, just 20 km away. Young children are known to be a source of respiratory viral transmission; therefore, our negative findings epidemiologically suggest that MERS-CoV may not have been widely circulating in nearby metropolitan Amman, Jordan, during this interval. This is an important finding because there remains uncertainty over whether confirmed MERS-CoV cases are more likely to be detected due to the severity of illness, and the full extent to which MERS-CoV circulates more widely causing mild illness [8]. Nevertheless, further surveillance is still needed in the Arabian Peninsula and neighbouring countries to monitor current circulation and possible emergence of widespread transmission. The majority of the MERS-CoV cases reported to date have been in adults and the common symptoms have included fever, cough and shortness of breath, with a high proportion of cases resulting in death [1,2,5,6,8].

Four specimens tested preliminarily positive for MERS-CoV by the upE rRT-PCR assay, albeit with high C_t values; however, further testing confirmed that these were false-positive results. This illustrates that with any assay, even in an experienced laboratory, false positives can occur. Thus, given the public health importance of this virus, further confirmatory testing should be performed on all provisionally test-positive specimens.

Our study is limited by the fact that only hospitalized children with ARI were enrolled, and thus we may have missed milder cases of MERS-CoV infection among outpatient children. For example, children < 12 years of age infected with SARS-CoV were reported to have milder disease compared with older children and adults [9,12,13]. Nose/throat swabs obtained during acute respiratory disease were tested, and the sensitivity of this specimen type for detecting MERS-CoV is currently unknown. The CDC recommends collecting respiratory specimens from different sites and at different stages of the illness, with lower respiratory tract specimens prioritized, but existing routine surveillance in the Middle East and elsewhere typically focuses on nose/throat swabs [5,14]. Only samples that tested negative for a virus were sent to the CDC for testing; therefore, we may have missed instances of MERS-CoV co-infections, but all respiratory samples collected after the first MERS-CoV cluster occured in Jordan in April 2012 were tested. However, a strength of our

study is that we tested almost 1000 children hospitalized with ARI symptoms for MERS-CoV. Of note, another study described lack of detecting MERS-CoV from nasal swabs from a group of French Hajj pilgrims after returning to France, despite a high rate of respiratory symptoms [15].

In conclusion, our negative findings suggest that MERS-CoV has not widely circulated in Amman, Jordan, during the 30-month period of prospective, active surveillance occurring before and after the first documented outbreak of MERS-CoV in the region. Our study confirms the value of prospective viral surveillance infrastructure to facilitate rapid responses to emerging pathogens.

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TABLE 1

Patient characteristics of the Jordanian hospitalized children < 2 years of age with fever and/or respiratory symptoms

	Tested for MERS at CDC *N* =474 (%)	Tested for MERS at Vanderbilt *N*=531 (%)	Not tested for MERS due to other virus detected *N* =1422 (%)	Combined population *N* = 2427 (%)	p-Value
Age (months)	2.8	3.2	3.8	3.5	<0.01
Sex (male)	297 (63)	339 (64)	830 (58)	1466 (60)	0.047
Prior medical history	54 (11)	70 (13)	114 (8)	238 (10)	0.001
Daycare	6 (1)	8 (2)	25 (2)	39 (2)	0.75
Exposure to smoke	371 (78)	403 (76)	1081 (76)	1855 (76)	0.57
Mother employed	26 (5)	30 (6)	96 (7)	152 (6)	0.5
Father employed	456 (96)	516 (97)	1375 (97)	2347 (97)	0.69
Number days sick prior to admission (median)	2.0	3.0	3.0	3.0	<0.01
Length of stay (median)	4.2	4.2	4.2	4.2	0.80
Diagnosis					
Pneumonia	30 (6)	71 (13)	168 (12)	269 (11)	<0.01
Bronchiolitis	34 (7)	50 (9)	328 (23)	412 (17)	<0.01
Bronchopneumonia	132 (28)	165 (31)	506 (36)	803 (33)	0.004
Sepsis ruled out	213 (45)	182 (34)	278 (20)	673 (28)	<0.01
ICU admission	49 (10)	49 (9)	134 (9)	232 (10)	0.81
Oxygen use	92 (19)	155 (29)	487 (35)	734 (30)	<0.01
Discharge status = death	7 (1)	5 (1)	10 (1)	22 (1)	0.31