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ORIGINAL ARTICLE

Role of Human Coronaviruses in Acute Respiratory Infections and Seroprevalence of Middle East Respiratory Syndrome Coronavirus in children

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ABSTRACT

Background: *Coronaviruses have been the focus of many studies since the emergence of SARS-CoV. Data on the role of MERS-CoV in respiratory tract infection and the seroprevalence of MERS-CoV in Egypt are limited.* ***Objective:*** *This study aimed to determine the role of coronaviruses in respiratory tract infections and the seroprevalence of MERS-CoV in pediatric age group.* ***Methodology:*** *Respiratory samples were collected from 80 children with respiratory infections for detection of coronaviruses using PCR technique. Serum samples were collected from 200 children for detection of MERS-CoV IgG immunolglobulins.* ***Results:*** *4 out of 80 (5%) of the respiratory samples tested positive for coronavirus (OC43 subtype). None of respiratory samples tested positive for MERS-CoV, while MERS-CoV IgG was detected in 1% of serum samples.****Conclusion:*** *A low prevalence of coronaviruses was observed in children with respiratory infection. A seroprevalence of 1% MERS-CoV was detected. Further studies are recommended on larger scale.*

INTRODUCTION

There are currently seven recognized types of coronavirus that can infect humans, Common types include 229E, NL63, OC43 and HKU1[1]. Less common, more dangerous types include Middle East Respiratory Syndrome Coronavirus (MERS-CoV), Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)[2]. Finally, the new strain of human coronavirus (SARS-CoV-2) that was declared as a global pandemic by the world health organization in 2020 and spread to many countries around the world including Egypt [3]. The emergence of such deadly strains brought the attention to Human Coronaviruses and has recently made them under focus.

Human Coronaviruses (HCoVs) account for up to 20% of upper respiratory tract infections in adults [4], and near 15.52% of acute respiratory illness in children[5]. They are also implicated in lower respiratory tract infections[6]. Children are found to be less affected by HCoVs causing severe respiratory syndromes, for example: MERS-CoV was found to be asymptomatic in 42% of affected children in Saudi Arabia (the mainland of the disease). Also, in the current pandemic of COVID-19, recent evidence suggests children to get infected as likely as adults but are less likely to be symptomatic or develop severe symptoms [7].

The emergence of MERS-CoV dates back to July 2012 when a new virus strain was isolated from a patient with severe respiratory tract infection in Saudi Arabia [8], followed by isolation of a virus from a Qatari patient in London with a homology of about 99.5 % in nucleotide sequence between the two viruses. The isolates were closely related to bat coronavirus (Bat-CoV), and according to the recommendations by the International Committee on Taxonomy of Viruses (ICTV), the new coronavirus was named as "Middle East Respiratory Syndrome Coronavirus" (MERS-CoV)[9].

Camels have been recognized as a source of infection of MERS-CoV [10]. In Saudi Arabia, the seroprevalence of MERS-CoV antibodies was significantly higher in camel-exposed individuals than in the general population [11].

From 2012 to 2015 in KSA, 1250 patients were reported to have MERS-CoV infection, and 3.3% of them aged less than 10 years with the first case in the pediatric age group reported on June 28, 2013 [12]. In 2019, Alfaraj et al[13] reported 7 new pediatric cases in Saudi Arabia of which 43% were asymptomatic and 14.3% required ventilator support and all of them were discharged without complications.

Limited data are available about the prevalence of the virus, and its antibodies in human in Egypt and almost all studies were performed on camels, and revealed high seroprevalence of MERS-CoV antibodies, especially in those imported from Sudan and East Africa [14, 15]. This indicated the ubiquitous presence of the virus in the country that warrants the initiation of active surveillance studies on humans. The present study

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aimed at recognizing Human Coronaviruses including MERS-COV that cause acute respiratory tract infections and estimating the seroprevalence of MERS-CoV in pediatric age group.

METHODOLOGY

An observational cross-sectional study was conducted on 200 children attending Ain Shams University Pediatric Hospital, divided into 2 groups: group I included 80 patients with respiratory tract infections, their ages ranged from 2 months to 15 years with mean (4.5 ± 3.5) with 46 males and 34 females. Group II included 120 patients attending the hospital for other medical reasons than respiratory tract infections, they were 60 males and 60 females and their ages ranged from 6 months to 17 years with a mean of (4.9 ± 5).

The aim and nature of the study were explained for each parent before inclusion. An informed written consent was obtained from parents or caregivers before being enrolled. The work has been carried out after approval of Ain Shams University Ethics Committee and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

Respiratory samples (nasal and throat swabs) were collected from Group I patients presenting with upper respiratory tract infection using sterile nasopharyngeal swabs, they were transported to laboratory in BioWhittaker® Dulbecco's Modified Eagle's Medium (Lonza, USA). Nasopharyngeal aspirates were collected using mucus traps from patients presenting with lower respiratory tract infection. All samples were kept at -80°C until processing.

Three milliliters of venous blood were taken from all study participants under complete aseptic conditions and were added to sterile empty tubes, allowed to clot for 30 min then centrifuged for 15 min at 1000g. Serum was then aliquoted and stored at -20°C until used in indirect immunofluorescence assay.

Detection of coronaviruses RNA by Pan-Corona nested RT- PCR:

RNA extraction: RNA extraction was performed using the QIAamp RNA minikit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's protocol. Extracted viral RNA was eluted in 60µl elution buffer and stored at -20°C

RNA amplification by nested Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): MyTaq™ One-Step RT-PCR Kit and MY TAQ master mix (Sigma-Aldrish, Germany) were used. A first round RT-PCR was carried out using sv387as and sv388s primers, followed by a second round (nested) of PCR using sv387as and sv389s primers [16]. The reaction mix was assembled into 50µL volume tube. Reverse transcription was carried out for 30 minutes at 42°C followed by a PCR with one initial step of Taq- Polymerase activation (95 °C for 15 min.), 35 cycles of amplification (95 °C for 30 sec., 50 °C for 30 sec., 72 °C for 30 sec) and a final elongation step (72 °C for 5 min) [16]. PCR-amplicons were electrophoresed on an agarose gel, and visualized by ethidium bromide. A known OC43 strain provided by prof. Ali Zaki, (Faculty of Medicine, Ain Shams University) was used as positive control and water used as negative control. Primers sequences and amplicon size are shown in table (1).

Genotyping of positive samples using type specific RT-PCR:

A one-step multiplex RT- PCR was performed for the positive samples for Pan-Coronavirus nested PCR using Qiagen one step RT-PCR kit (QIAGEN, Hilden, Germany) and 4 pairs of primers specific for the four main circulating types (OC43, NL63, HKU1 and 229E)[17]. The reaction mix was assembled into 50µL volume. Reverse transcription was carried out for 30 minutes at 50°C followed by a PCR with one initial step of denaturation (95 °C for 15 min), 40 cycles of amplification (95 °C for 30s, 58 °C for 30s, 72 °C for 1 min) and a final elongation step (72°C for 10 min)[18]

The 4 positive samples were also subjected to a one step RT-PCR using Qiagen RT-PCR kit (QIAGEN, Hilden, Germany) and primers specific for MERS-CoV. The reaction mix was assembled into 50µL volume. reverse transcription was carried out for at 45 °C for 10 min followed by followed by a PCR with one initial step of denaturation 95 °C for 2 min , 35 cycles of amplification (95°C for 10s ,60°C for 20s 72 for 1min) and a final elongation step 72 for 10 mins [19]

PCR-amplicons were electrophoresed on an agarose gel and visualized by ethidium bromide. Positive controls were provided by prof. Ali Zaki, (Faculty of medicine, Ain Shams University), and water was used as negative control.

The sequences of primers used for coronavirus type specific RT-PCR and the amplicon sizes according to Vabret et al [17] and Noh et.al [19] are described in table (2).

Table 1: The primers sequences and amplicon size of the Pan-Coronavirus nested PCR

	Primers	Amplicon sizes
First round	▪ sv387as (5'-TCACA TTGGATATCCCA) ▪ sv388s (5'-ACTCAATAATCTTAATAGC)	
Second round	▪ sv387as (5'-TCACATTGGATATCCCA) ▪ sv389s (5'-ACTCAAATAATTTAATAGC)	**251 bp**

Table 2: The primers sequences used for coronavirus type specific PCR

Type	Primers	Amplicon sizes
OC43	▪ MF1: (5′- GGCTTATGTGGCCCCTTACT-3′) ▪ MF3: (5′- GGCAAATCTGCCCAAGAATA -3′)	334 bp
229E	▪ MD1: (5′ TGGCCCCATTAAAAATGTGT -3′) ▪ MD3: (5′- CCTGAACACCTGAAGCCAAT -3′)	574 bp
HKU1	▪ HKU1 sense: (5′- ACCAATCTGAGCGAAATTACCAAAC-3′) ▪ HKU1 antisense: (5′- CGGAAACCTAGTAGGGATAGCTT -3′)	443 bp
NL63	▪ N5-PCR2: (5´- GATAACCAGTCGAAGTCACCTAGTTC-3´) ▪ N3-PCR2: (5- ATTAGGAATCAATTCAGCAAGCTGTG-3´)	255 bp
MERS CoV	▪ MCOV-F: (5´- CAG ACA ACC ATT CAG AAR GTT A-3´) ▪ MCOV-R: (5´- TTT AGA ACA AAA CTG GCC ATA-3´)	108 bp

Detection of IgG antibodies against MERS-CoV (S) antigen using Indirect-immunofluorescence test in patients' sera:

MERS-CoV IgG was detected in patients' sera using Fluorescein isothiocyanate (FITC) labeled Antihuman IgG (Sigma-Aldrish, Germany), and slide fixed with MERS-CoV (S) antigen (provided by prof. Ali Zaki - Faculty of Medicine, Ain Shams University). Briefly, a 1/10 dilution solution was prepared for each serum sample using 30µl of each serum sample and 270µl Phosphate Buffer Saline (PBS). The slides with fixed MERS-CoV (S) antigen were labeled and 25µl of each diluted serum sample were placed in each of the wells, conserving 2 wells in each slide for control. 25µl of Fluorescein isothiocyanate (FITC) labeled Antihuman IgG were added and slides were incubated at 37°C in the moist chamber for 1 hour then they were washed, left to dry and finally examined under florescent microscope.

Statistical analysis:

All collected data were statistically analyzed by appropriate statistical methods (Statistical Package for the Social Sciences SPSS-20)

Numerical data were expressed as mean, standard deviation and percentage; non-numerical data were expressed as frequency and percentage

RESULTS

The present study comprised 200 pediatric patients attending the pediatric Ain Shams University hospital, they were divided into 2 groups: group I included 80 patients with respiratory tract infections. Group II comprised 120 patients attending the hospital for other medical reasons than respiratory tract infections.

Group I was further divided into: Group IA, included 53 patients presented with symptoms of upper respiratory tract infections. Group IB comprised 27 patients with lower respiratory tract infections. Table (3) summarizes the demographic and clinical data of group I patients.

Table 3: Demographic and clinical data of group I patients

Demographic and clinical data:	**Group IA No.=53**	**Group IB No.=27**
Age: (mean ±SD)	(4.3 ± 2.9)	(5.1 ± 4.1SD)
Sex:		
▪ Male	31(58.4%)	15 (55.5%)
▪ Female	22 (41.5%)	12 (44.4%)
Symptoms		
▪ Fever	48 (90.5%)	27 (100 %)
▪ Cough	17 (32%)	21 (77.7%)
▪ Wheezes	7 (1.8%)	4 (14.8%)
▪ Expectoration	5 (9.4%)	19 (70.3%)
Associated comorbidities	0 (0%)	11 (40 .7 %)
▪ Bronchial asthma		4 (36.3%)
▪ Renal disease		2 (18.2 %)
▪ Congenital heart disease		2 (18.2%)
▪ Cerebral palsy		3 (27.2 %)
Repeated hospital admission:		10 (12.5 %)
Laboratory findings:		
▪ CRP		18 (66.6%) negative 9 (33.3%) positive (mean: 16.3± 3 SD)
▪ TLC		15 (55.5%) normal 12 (45.5%) high (mean:13.3±1.2) $x10^{\underline{9}}$ cells/L
Outcome		
▪ Ventilator support		15 (55.5 %)
▪ Recovery	53 (100%)	20 (75%)
▪ Death	0%	7 (25 %)

Out of the 80 patients, 4 patients (5%) were positive for coronavirus RNA by the pan- coronavirus RT-PCR. All the positive cases belong to group IA patients. Figure (1) shows the photo of gel electrophoresis of the product of the Pan-Coronavirus nested PCR.

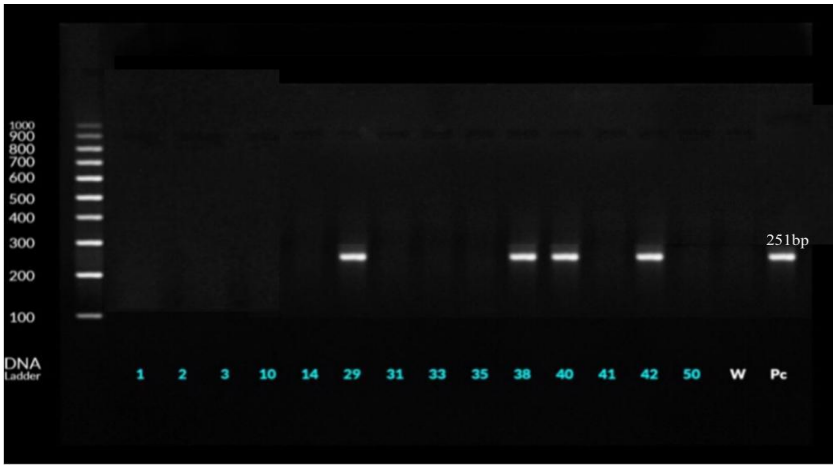

Fig. 1: Pan-coronavirus nested PCR, positive bands are detected at 251 bp in samples number 29, 38, 40 and 42 *Pc: positive control *W: water used as negative control

The 4 coronaviruses isolated from the patients were further subtyped using type specific multiplex PCR. They all belonged to the subtype OC43. **Figure (2)** shows the photo of gel electrophoresis of the products of the type specific multiplex PCR.

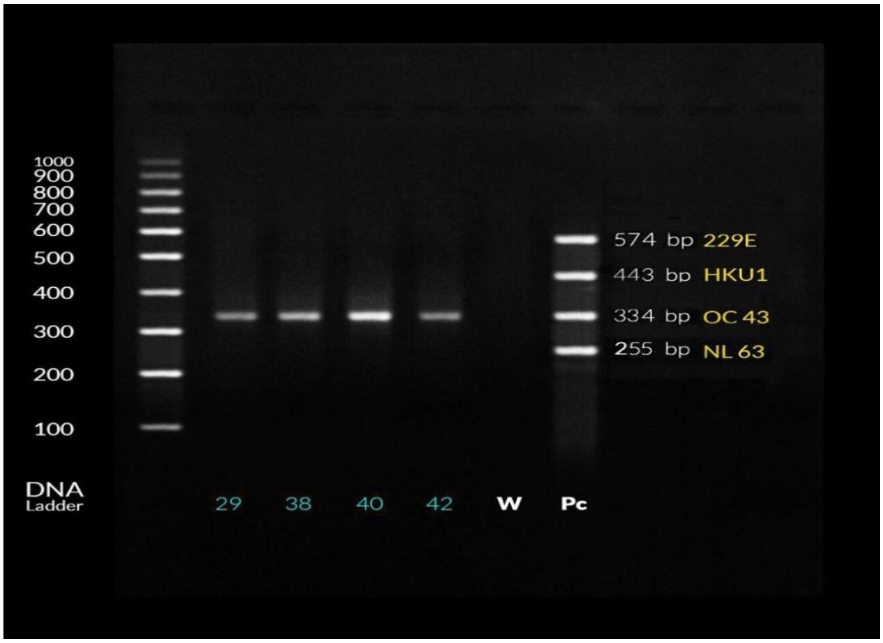

Figure 2: Coronaviruses type specific PCR. Positive bands were detected at 334 bp (OC43 subtype). *Pc: positive controls *W: water used as negative control

Out of 200 serum samples tested, 2 samples (1%) tested positive for MERS-CoV IgG by Indirect Immunofluorescence (IIF) technique. Both positive samples belong to patients of group II. None of the children of group I tested positive for MERS-CoV IgG.

DISCUSSION

Human Coronaviruses (HCoV) are found to occur in 1 of 10 hospitalized children with respiratory tract infections [20] and are responsible for 15.52 % of acute respiratory illness in children [5].

The emergence of the highly pathogenic strains including SARS-CoV, MERS-CoV and now SARS-CoV-2 have driven HCoVs into the center of the scientific researches [2].

To the best of our knowledge, our study is the first to investigate MERS-CoV as a cause of respiratory tract infections in the Egyptian children.

Our results revealed a 5% prevalence of Coronaviruses among children presenting with acute respiratory tract infections. This prevalence was close to that reported in previous studies that detected HCoV infection in 4.1%, 4.4% and 6% of children presenting with acute respiratory tract infections respectively[21,22,23].

A higher percentage of coronavirus infection (9.1%) was detected in hospitalized children in Italy in a long term 3-year study conducted on 3458 children [20].

MERS-CoV RNA was not detected in any of this study population. These results were similar to a study conducted in Jordan during April 2012 in an area about 20 Kilometers away from Zarga, where an outbreak of MERS-CoV occurred, they reported that MERS-CoV was not detected among children hospitalized with acute respiratory tract infection [24]. This can be explained by the fact that children are more likely to be asymptomatic when infected with the virus as reported by two major studies conducted in Saudi Arabia, in which children infected with MERS-CoV were found to be asymptomatic [25,26].

Human Coronavirus-OC43 was detected in all (4) positive samples, in the present study, suggesting its predominance in Egyptian population. This is supported by the finding that NL63 and OC43 are the most frequently isolated species, with almost all surveillance data indicate HCoV-OC43 as the most common species and HCoV-229E as the least common among the four common types [7]. Similar to ours results, Naga et al [27], in their study conducted on 100 Egyptian children in Alexandria, reported that OC43 was detected in 3% of samples. Outside Egypt, the predominance of OC43 was also declared in Norway by Heimdal et al [20]. A study conducted in Slovenia found HKU1 to be predominant[28]. NL63 was detected in 28 (9.3%) of the 300 samples in a study conducted among children in France[18].

Such differences in the isolated types of coronaviruses and their role in respiratory tract infections can be explained by the fact that the frequency of detection of the four major strains varies by geography and over different seasons. Another explanation is due to the cyclical patterns that have been observed in many studies for 229E and OC43, with

outbreaks occurring every 2–4 years. Seasonal patterns have also been detected, in the Northern Hemisphere, HCoVs mostly cause infections in humans between December and May, and in the Southern Hemisphere between March and November with peaks in late winter/early spring for 229E and OC43 and in autumn for NL63 [7].

None of the patients with lower respiratory tract infection in our study was positive for coronavirus RNA. This result was in contrast to many previous studies. Meligy et al [29] reported that HCoV was isolated from 4.5% of the nasopharyngeal aspirates collected from their studied group of children with lower respiratory tract infection. Another study in China reported a 2.3% rate of detection of HCoV in nasopharyngeal samples collected from 659 hospitalized children (2 months to 14 years) with severe lower respiratory tract infections [30].

In a study conducted in Canada, Jean et al [31] did not detect Coronavirus infection in children with lower respiratory tract infections, who had associated comorbidities. In contrast to their results, Vabret et al [17] found that One third of HCoV-infected children suffered from underlying chronic diseases.

The results of the present study were affected by some limitations including the small number of our studied patients groups with the lack of surveillance over a prolonged period of time, which could affect the results in case an outbreak of a specific virus occurred in a given period time.

The increasing MERS-CoV seroprevalence was observed in the general population in western Saudi Arabia, suggesting that asymptomatic or mild infections might exist and act as an unrecognized source of infection. There was also seropositivity among individuals from other countries including Egypt, Yemen, Pakistan, Palestine, Sudan, and India, raising the potential of MERS-CoV exportation outside of the Arabian Peninsula. Such data raised the need for surveillance of MERS-CoV around the world [32].

In addition, serology is considered to play an important role in understanding the impact of any infection at the level of a given population**.** Many studies have been conducted to investigate the seroprevalence of MERS-CoV around the world. However, most of them were conducted in camels in many countries including Egypt [14, 15, 33, 34]. Very few studies were conducted in humans, most of which belong to the mainland of the disease (Saudi Arabia) [2, 35]**.** Serological diagnosis of MERS-CoV infection is performed by using ELISA or Indirect immunofluorescence (IIF) for screening of samples. Positive samples can be confirmed using a plaque reduction virus neutralization test (PRNT) [36].

To our Knowledge, there are no reports of investigating MERS-CoV seroprevalence in humans in Egypt, except for one study conducted in the Faculty of Veterinary Medicine, Assuit University. They tested serum samples of a group of camels and their traders for MERS-CoV antibodies. They reported that 58.73% of imported camels and 25% of traders had antibodies specific to MERS-CoV [37]**.**

In the present study, 200 serum samples from children were tested for anti S antigen MERS-CoV IgG. Only 2 samples out of 200 (1%) tested positive using IIF. Our results were consistent with a study conducted in Kenya that showed 1.43% (16 out of 1122) seroprevalence of MERS-CoV using ELISA on serum samples of a group of patients ranging from 5 to 27 years old [38]. Another study conducted in Saudi Arabia showed that MERS-CoV seroprevalence in the area around King Fahd Hospital was less than 2.3% in pediatric age group when a study investigating the presence of MERS-CoV neutralizing antibodies was conducted on 158 serum samples from children during 2012 [39].

In their study, Degnah et al [32] found that 2.5% out of 7461 tested serum samples, were ELISA positive for MERS-CoV IgG antibodies using ELISA. Indirect ELISA results showed that the seroprevalence of MERS-CoV for the years 2011, 2012 and 2013 were 1.11%, 0.74% and 1.8%, respectively with an increased seroprevalence of 3.01% in 2016.They also reported that only 0.23% were confirmed positive by neutralization test.

The IIF results of the present study suggest a low prevalence of MERS-COV among Egyptian pediatric group. However, the serum samples found positive by the IIF needed to be confirmed by the gold standard neutralization test to rule out any cross-reactions that may occur between different HCoV strains. Such gold standard neutralization methods require biosafety level-3 laboratories; they are labor-intensive and time-consuming process that requires at least 5 days before results are available [40].

CONCLUSION

The present study is considered a preliminary step in the way to investigate the prevalence of coronaviruses with emphasis on MERS-COV among Egyptian pediatric population. The results of this study suggest a very low seroprevalence of MERS-CoV in Egyptian children and a low prevalence of coronaviruses among children with respiratory tract infection. Further studies among larger groups of children over longer periods of time and from different governorates will help understand the true impact of MERS-CoV among pediatric population in Egypt.

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Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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