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Molecular Detection of Respiratory Syncytial Virus in Infants and Young Children by the Conventional Reverse Transcriptase Polymerase Chain Reaction

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ABSTRACT

Background: At the peak of the viral season, World Health Organization ranked respiratory syncytial virus (RSV) as a major cause of acute respiratory infections in more than 60% of children and more than 80% of infants younger than one year. The current study is the first in the governorate of Was it to investigate respiratory syncytial virus subtypes. The objectives of the study were to estimate the frequency of respiratory syncytial virus in children ≤5 years old and to recognize some potential risk factors that might be associated with respiratory tract infections.

Methodology: A cross sectional study with conventional reverse transcription polymerase chain reaction was performed on nasopharyngeal swabs from 158 pediatric patients. We compared the demographic and clinical characteristics of the patients aged 15 days to 60 months hospitalised with RTIs or seen at private clinics (cases, n = 158) and control children (n = 40) with non-respiratory symptoms during the respiratory season of 2021–2022. Pearson's chi-square (x^2) model was applied, and $P \leq 0.05$ was considered significant.

Results: RSV nucleic acid was detected in 15 (9.49%) of the 158 clinical specimens after amplification of the F gene. The identity of these amplified fragments was confirmed as human respiratory syncytial virus subtype B by sequencing. Except the clinical presentation there was no overall association between negative and positive cases while breast feeding and family history of the same condition when comparing the control and positive cases showed statistically significant.

Conclusions: conventional PCR was successfully detected the subtype B of h RSV while it may not the appropriate PCR type for subtype A detecting or that the RSVB was the only subtype circulated in 2021-2022 winter.

Keywords: *RSV, Respiratory tract infection, Demographic, Clinical characteristics, Iraq*

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INTRODUCTION

Viral respiratory infections have been identified as the most common cause of childhood morbidity and mortality, particularly within the developing countries (1). Most of this disease burden occurred in children younger than five years old (2). Respiratory Syncytial Virus (RSV) has been recognized as one of the commonest causes of childhood acute upper and/or lower respiratory illnesses which responsible for about 50% to 90% of bronchiolitis cases and about 50% of pneumonia cases among the pediatric population worldwide (3-5). Nearly 45% of these cases require hospital admission with most death occur in infants less than 6 months of age (6). RSV infection in children is almost always symptomatic, illness ranging from barely notice, or mild nonspecific symptoms to severe and sometimes fatal disease of the lower airways (7).

Nearly 70% of infants have been infected with RSV at least once by their first birthday, and nearly all children are infected with the virus within the first two years of life (8). In developing countries, reinfection rates with RSV ranging from 6% to 83 % each year and tend to be milder (9, 10). Despite the presence of maternal antibodies, RSV can reinfect infants due to antigenic variability or inadequate immune response (11). However, as of May 17, 2022, FDA had not approved any RSV vaccine; however, there is an authorized passive antibody therapy, Palivizumab.

Concerning the virology of the virus, RSV is enveloped, negative-sense, non-segmented, single strand RNA virus (-ssRNA) of the family Pneumoviridae. The genome is approximately 16 kb and is comprised of 10 genes encoding for 11 proteins (12). RSV has been genetically classified into two subgroups, A and B; and there are numerous genotypes within each of these two subgroups, to date, 15 genotypes of RSV-A and 37 genotypes of RSV-B are discovered (13, 14) and these genotypes have been classified according to the analysis of nucleotides sequence of glycoprotein (G) gene (15), these subgroups not only predominate alternately but they can co-circulate at the same season (16, 17).

Clinical microbiology laboratories play an important role in the diagnosis and control of infectious diseases. However, the ability of laboratories to perform these tasks is limited by the quality of samples collected from patients, the manner in which samples are transported from patients to laboratories, and the techniques used to detect microorganisms in samples. The accuracy of RSV infection and rapid laboratory confirmation are essential to initiate early treatment, reduce antibiotic use, prevent viral transmission, minimize unnecessary diagnostic tests and interventions, and shorten hospitalisations stays (18).

METHODOLOGY

Samples collection

Nasopharyngeal and/or oropharyngeal swabs were collected from 158 patients ≤5 years old diagnosed at time of consultation regarded as respiratory tract infection by pediatrician from the in- and outpatient ward of Al-Kut Maternity and Child Hospital, Al-Karama Teaching Hospital, and Al-Zahra'a General Teaching Hospital or those who were seen at outpatient clinicsduring the period of respiratory season (November to February) in the winter of 2021 to 2022 at Wassit Province, south of Iraq. These hospitals are the largest hospitals located in the city center treating patients from rural and urban areas. Moreover, nasopharyngeal and / or oropharyngeal swabs were collected from 40 asymptomatic infants and children under the age of five with no recent history of respiratory tract infections. Specimens were collected during the same period.

After consent was obtained from participants' families, specimens were collected with a swab from both the nose and the throat of the patients using flocked-fiber nasal and throat swabs (Biobase, China), which consist of a flocking fiber head that can effectively absorb samples to achieve the purpose of collection and an Acrylonitrile Butadiene Styrene rod that can effectively protect the target tissue from injury and according to the manufacturer's instructions. These swabs were immersed in 3 ml of viral transport medium (VTM) and kept at –20 until use. Each specimen was collected with a standardized questionnaire filled out from medical files and the parents of the participants' children in this study.

Molecular examination

Viral nucleic acid was extracted from 150 µl of the clinical specimens using the Viral Gene-spinTM Viral DNA/RNA Extraction Kit (Intron/Korea), according to the manufacturer's instructions.

Conventional PCR technology was done as two steps reverse transcriptase PCR, since the RSV sought to be detected is an RNA virus so conversion of RNA to complementary DNA is essential for amplification. AddScript cDNA Synthesis Kit (Addbio / Korea) offers sensitive and simple-to-use componentswas chosen for this study. This kit includes thermostable (Molony-murine leukemia virus MMLV) enzyme for reverse transcription and RNase Inhibitor to prevent RNA degradation included in 20x AddScript Enzyme Solution. cDNA synthesis protocol was as follow, 20 µl of total reaction volume contain 4 µl of RNA template, 2 µl of dNTP Mixture, 2 µl of oligo dT20 [or 10x random hexamer], 1 µl of AddScript Enzyme Solution, 1 µl of Nuclease-Free H2O and 10 µl of Reaction Buffer were added to a thin-walled PCR tube. the following temperature cycling Protocol were as follow: at 25°C for 10 min for priming, 50° C for 60 min for reverse transcription, 80°C for 5 min for RT inactivation followed by hole in 12°C. The cDNA samples were stored at 20°C for further use.

Amplification by PCR of the partial region of the F gene were performed using PCR and sequencing primers modified from Song et al. (19), (Table 1). A total of 50 µl of reaction mix contained 4 µl cDNA, 25 µl GoTaq® G2 Green Master Mix, 13 µl ddH2O and 4 µl of each forward and reverse primers. The lid of the PCR thermocycler (Eppendorf- Mastercycler gradient) was preheated at 95°C before starting the amplification steps. The optimization was conducted at 56°C and the PCR conditions were as follow: 94°C for 5 min for Initial denaturation, and with 40 cycles of 94°C for 30 sec for denaturation, 50-58°C for 30 sec for annealing, 72°C for 1 min for extension, followed by 72°C for 5 min for a final extension.

TABLE 1: Primers used in this study.

Organism	Primer name (5'-3')		Size (bp)	Access No.	Reference
RSVA human	RSVA-F	TCCAGAACWCAC AAGTCAA	683	MG642060	Song *et al*. (2018)
	RSVA-R	CAGGACYTTRGAT ACAGCAA			
RSVB human	RSVB-F	TCTTCCTAACTCTT GCTATT	1040	OK649754.1	
	RSVB-R	AGTGTCAGCYTGT GGAAAGA			

The electrophoresis has been performed to visualise the PCR product size after finishing the PCR programme. In this study the concentration of the gel was 1.5% for the PCR products. About 5 µl of each PCR products were inserted into the middle of it correspondence hole in the 1.5% gel. About 5 µl of Safe-Green 100bp Opti-DNA Marker was added to the first hole in the lines of the gel to be served as a marker for measuring the size of the PCR products. Following the loading of Safe-Green 100bp Opti-DNA Marker and the samples, the electrophoresis system was set as following: 90 Volt, constant current, 45 minutes time. Finally, the gel was transferred into UVP system to visualize the PCR products under 320nm UV light source.

Statistical Analysis

Data of the 198 participants were analyzed using SPSS version 23.0. Continuous, non-normally distributed data were expressed as median and range. Associations between the groups were analyzed using the Pearson Chi-square (x^2) and differences were considered significant at p-value of <0.05 ().

RESULTS

The current study was carried out on 198 participants (158 patient group and 40 controls with non-respiratory illness), admitted to pediatrics department of three major hospitals, or seen at private clinics during a period from November 2021 to February 2022. The RSV ribonucleic acid detected in 15(9.49%) of the 158 patients studied with a PCR product of approximately 1040 bp only. This observed size corresponded to the expected size of RSV-B. Four were select randomly for validation the conventional PCR products of representative samples identified as positive hRSV and the identity of these amplified fragments was confirmed as human respiratory syncytial virus subtype B by sequencing.

TABLE 2: Number of children studied and percentage of hRSV by PCR

Groups	No. of children	Positive cases	Negative cases
Patients	158	15 (9.49%)	143 (90.50%)
Control	40	0 (0%)	40 (100%)
Total	198	15 (7.58%)	183 (92.42%)

Demographic and clinical data of the suspected patients were compared with the negative and positive cases and shown in Table 3. The majority of the participant were below 12 month of age, 69(43.7%) of them were younger than 6 months old, the median age was 8 months (ranging from 14 days to 5 years). Among the 158 pediatric patients, 81(51.3%) were males. History of prematurity found in 16(10.1%) patients, type of feeding was formula-fed 63(39.9%), and household smoker found in 83(52.5%). Totally, 70.9% of the patients were reported to have a family history of the same condition in the last 2 weeks. 112(70.89%), were managed as inpatients. 92(58.23%) were living in the city center.

The clinical presentation of the patients was as follows: 32(20.3.1%) had cough only, 32(20.3.1%) had fever and cough, 58(36.7%) had fever, rhinorrhea, and cough, and 36(22.8%) experienced rhinorrhea and cough. Among the clinical diagnoses of the patients 30(19.0%) had acute bronchitis, 95(60.1%) had bronchiolitis, 26(16.5%) had pneumonia, and 7(4.4%) had unclassified LRTIs.

TABLE 3: Association of demographic and clinical data according to PRC results

Item	No. (%) =158	Negative=143	Positive=15	*P* value
Gender				
Male	81(51.3)	73(46.2)	8(5.1)	0.5
Female	77(48.7)	70(44.3)	7(4.4)	
Address				
Center	92(58.2)	80(50.6)	12(7.6)	0.7
Periphery	66(41.8)	63(39.9)	3(1.9)	
Age (months)				
≤ 6	69(43.7)	61(38.6)	8(5.1)	0.3
6 - 11	32(20.3)	29 (18.4)	3 (1.9)	
12 - 24	20(12.7)	17 (10.8)	3(1.9)	
25 - 60	37(23.4)	36(22.8)	1(0.6)	
Family history of smoking				
Smoking	83(52.5)	74(46.8)	9 (5.7)	0.3
No Smoking	75(47.5)	69(43.7)	6(3.8)	
Type of Feeding				
Breast fed.	20(12.7)	19(12)	1 (0.6)	0.8
Formula	63(39.9)	56(35.4)	7(4.4)	
Mixed	41(25.9)	37(23.4)	4(2.5)	
Family food	34(21.5)	31(19.6)	3(1.9)	
Family history of same condition last 2 weeks				
No family history	46 (29.1)	40(25.3)	6(3.8)	0.2
Family history	112(70.9)	103(65.2)	9(5.7)	
History of prematurity				

Mature	142(89.9)	130 (82.3)	12 (7.6)	0.1
Premature	16(10.1)	13 (8.2)	3(1.9)	
Patient management				
In patient	112(70.9)	103(65.2)	9(5.7)	0.3
Out patient	46(29.10)	40(25.3)	6(3.8)	
Clinical presentation				
Cough	32(20.3)	30(19.0)	2(1.3)	
Fever, Cough	32(20.3)	32(20.3)	0(0)	0.01
Fever, Rhinorrhea, Cough	58(36.7)	53(33.5)	5(3.2)	
Rhinorrhea, Cough	36(22.8)	28(17.7)	8(5.1)	
Clinical diagnosis				
Acute Bronchitis	30(19.0)	28(17.7)	2(1.3)	0.5
Bronchiolitis	95(60.1)	84(53.2)	11(7.0)	
Pneumonia	26(16.5)	25(15.8)	1(0.6)	
Unclassified LRTI	7(4.4)	6(3.8)	1(0.6)	

TABLE 4: Association of demographic data in patient and control

Item	Control =40	Positive =15	*P* value
Gender			
Male	20(36.4)	8 (14.5)	0.5
Female	20(36.4)	7 (12.7)	
Age (months)			
≤6	10 (18.2)	8 (14.5)	0.1
6-11	10(18.2)	3 (5.5)	
12-24	9 (16.4)	3 (5.5)	
25-60	11 (20.0)	1 (1.8)	
Family history of smoking			
Smoking	16 (29.1)	9 (16.4)	0.1
No smoking	24 (43.6)	6 (10.9)	
Type of Feeding			
Breast fed.	14 (25.5)	1 (1.8)	0.04
Formula	7 (12.7)	7 (12.7)	
Mixed	8 (14.5)	4 (7.3)	
Family food	11 (20.0)	3 (5.5)	
Family history of same condition last 2 weeks			
No family history	34 (61.8)	6 (10.9)	0.003
Family history	6 (10.9)	9 (16.4)	
History of prematurity			
Mature	39 (70.90)	12 (21.82)	0.1
Premature	1 (1.8)	3 (5.45)	

DISCUSSION

In response to the fact that respiratory infections are highly witnessed in winter and the peak of bronchiolitis happening at the same period, moreover, the absence of routinely diagnosis of RSV in hospitals and laboratories and poor information and experience of diagnosing this virus represent a reason for conducting this study.

This study was designed to detect hRSV in children aged ≤5 years old and conventional RT-PCR assay has been employed to achieve this goal. Conventional PCR technology has advantages over real-time since it provided an opportunity for PCR products to be sequenced, giving additional information that would be significant for patient managements or public health purposes. Also, it is an appropriate qualitative diagnosis that can also be used as an alternative to quantitative PCR, owing to the high costs of reagents and instruments for qPCR.

After the amplification of a partial region of the F gene using the primers mentioned above in table one,RSVB was detected in 15(9.49%) of the clinical specimens which was less than the 36% reported in Baghdad (21), and less than the 18.75% reported in Wassit (22) and the 20.4% reported in Kurdistan region (23) and close to the 10.8% reported in India (24) andhigher than 6.6% reported in Baghdad, Iraq (25).

Many new studies have highlighted the very low rates of RSV infection in children, resulting in a nearly complete absence of both RSV and influenza in the last two years during the COVID-19 pandemic (26-29). This high reduction in the last two years compared to pre-pandemic could be explained by the fact that the mitigation efforts taken to limit the COVID-19 pandemic such as habits of using antiseptics and nonpharmaceutical interventions (NPI) mainly hand sanitizers and face masks led to a slowdown in the spread of respiratory viruses sharing the same mode of transmission such as RSV and influenza and reducing their epidemic peak (30, 31).

Among other explanations contributing toRSV reduction could be viral interference, it has been recognized that the influenza virus can interfere with other respiratory viruses, and it's possible that SARS-CoV-2 and RSV pass through a similar interaction (32). The low rate of co-infection between SARS-CoV-2 and other respiratory viruses adds support to this hypothesis (33). However, still we can see respiratory tract infections in children and the related potential explanation could be due to the dissemination of the non-enveloped viruses. Authors suggest that despite the sanitary measures to control SARS-CoV-2 transmission, rhinoviruses, coxsackievirus A and B, and adenoviruses have not been affected by hygiene measures since they are non-enveloped and naturally more resistant to being destroyed by an ethanol-containing disinfectant (34-36).

Upon analyzing the patient population by gender type, numbers of male and female patients were 81 (51.3%) and 77 (48.7%), respectively. The male to female ratio was 1.05:1. Of the male patient population, 8(53.3%) were positive for RSV, while 7(46.7%) of the female patient population were positive for RSV. In line with other studies that have been published, gender was not found to be a significant risk factor for RSV infection in our study (36-38). However, certain research results, as reported by D'Elia et al. (39) have indicated a male predominance.

Concerning the effect of age on RSV infection, the median age of the positive cases in this study was about 6 months, and 11/15 (73.3%) of the RSV positive children were younger than one year old, as was to be expected. This was similar to what has been reported elsewhere (40-42) which confirmed an increase in RSV hospitalizations with a decrease in patient age, with RSV admissions peaking within the first year of life. This could be attributed to underdeveloped immune systems, weaned trans-placental maternal antibodies, and tiny airways so they are more susceptible to physical obstruction (the average bronchiole diameter in 2-4-month-old newborns is 120 m, compared to 250 m in an adult (43).

Regarding feeding, breast feeding of babies with acute viral respiratory tract infections showed a significant effect, which is consistent with most published studies (44, 45), which confirm that infants with breast feeding have a lower number of RSV associated hospitalizations and a reduced risk of respiratory failure compared to infants without breast feeding and being bottle fed (46). Breast milk contains many protective factors, such as lactoferrin, immunoglobulins, and lymphocytes, as well as other factors that may help reduce infant mortality, through either transfer of maternal antibodies or enhancement of virus-specific lymphocyte transformation activity (47).

Other studies have demonstrated that the presence of a family member with the same disease increases the likelihood of contracting the virusso this is another consideration when determining the likelihood of viral transmission (31, 48, 49). In this study, among cases, approximately 70% of all household members had respiratory symptoms at enrollment and over half, 60%, of the RSV-infected patients were lived with family members sharing the same respiratory symptoms, this indicate that possible transmission could occur between an infected family member and children within the home p value 0.003.

The increased risk due to increased proximity is greater during the first few months of life and may be offset by development of the immune system and other long-term immunomodulatory effects of breast milk that have not yet been elucidated (50). Adults infected with RSV can easily transmit the virus to children or other adults (51). This could arise the hypothesis that adults are the major source of viral transmission to children, therefore, blocking viral transmission to children, for the most part, depends on hygiene and behavioral measures of adults.

In this study, at least one family member was a smoker in more than half 83(52.5%) of the families. RSV frequency increased in patients that were exposed to smoking more than in patients who were not exposed; 9(60%) and 6(40%), respectively. Despite the fact that this exposure had an effect on RSV-infected patients, there was not a statistically significant difference between exposed and non-exposed patients p value 0.1. Similar results were reported by other authors who demonstrated that children who exposed to smoking have been hospitalised for severe RSV infections suchas sudden infant death syndrome (SIDS), wheezing, and asthma (52, 53). Thelink between smoking and RSV appears to be due to nicotine's effects on lung development (54). Moreover, a systemic review by DiFranza et al. (2012) demonstrated that exposure to cigarette smoke, in general, seems to worsen the severity of RSV infection (55).

Among the clinical presentation and diagnosis of positive cases, the clinical symptoms such as cough, fever and rhinorrhea were reported in most children infected by different viruses which make it difficult for the clinician to draw an accurate diagnosis. Also, RSVwas found to be responsible for 11/15 (73.3%) of the bronchiolitis cases, followed by 2/15 (13.3%) of the acute bronchitis cases and 1/15 (6.7%) of pneumonia cases. Moreover, RSV was detected in 1/7(6.7%) sample of patients with unclassified LRTIs. This was in agreement with what were published as Cunningham (2019) who reported that bronchiolitis is typically caused by RSV in about 70% of cases (56). Another study by Goto-Sugai et al. (2010), reported that among the clinical diagnosis of the patients, bronchiolitis was the most prominent sign for RSV infection especially in infants (57). Also, Tabatabai et al. (2022) was reported that the majority of RSV positive cases were presented with bronchiolitis (58).

CONCLUSIONS

Conventional PCR was utilized as the diagnostic tool for detecting human respiratory syncytial virus, and sequencing was performed for validation of the amplified fragments obtained from positive specimens as human respiratory syncytial virus subtype B. Subtype A disappearance could mean that the B subtype was the only one circulated, or it could mean that the primer attachment site of the subtype A has altered so significantly or that there is a local sequence that is not being picked up by these primers that can muddle a conventional PCR test and give negative results.

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