http://dx.doi.org/10.4314/bajopas.v15i1.22

Bayero Journal of Pure and Applied Sciences, 15(1): 152 - 157
Received: February, 2022
Accepted: April, 2022
ISSN 2006 – 6996

MOLECULAR DETECTION OF RESPIRATORY SYNCYTIAL VIRUS IN CHILDREN ATTENDING SELECTED HOSPITALS IN KADUNA STATE, NIGERIA

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ABSTRACT
A serological survey was carried out among children in two hospitals within Kaduna, Kaduna state to determine the level of Respiratory syncytial virus (RSV) IgM antibodies. Blood samples and nasal swabs of children aged 0-60 months were collected in two hospitals in Kaduna metropolis (Barau Dikko Teaching Hospital and Yusuf Dantsoho Memorial Hospital), Kaduna state. Respiratory Syncytial Virus IgM antibody level was measured using commercial ELISA kit obtained from DEMEDITEC DIAGNOSTIC GMBH Germany. The overall prevalence of RSV out of 192 samples of both blood and nasal swab samples collected 56.1 % positive for all the samples from both hospitals. Barau Dikko positive samples recorded 26.0 % and Yusuf Dantsoho 30.0 % as shown in figure 1. More so, the distribution of RSV with regards to socio-demographic characteristics, males had a higher predominance 31.0 % than females 26.0 % but the result was not statistically significant (p value= 0.285). Infants under one month had the highest prevalence rate of 75 % while 31-61 months had the least prevalence rate of 11.0 %. The difference was statistically significant (p value= 0.001). Respiratory syncytial virus RSV-G gene was detected among IgM seropositive children using a molecular analysis. Conclusively, Polymerase chain reaction on agarose gel electrophoresis verified the molecular characteristics of the RSV-G gene, which showed a typical band size of 326bp.

INTRODUCTION

Human respiratory syncytial virus (HRSV) is a single-stranded RNA virus belonging to the Paramyxoviridae family, with ten genes encoding eleven proteins (Marie *et al.,* 2008). The main viral antigens, the F (fusion) protein and the G (attachment glycoprotein) protein, are both surface proteins that play a key role in RSV virulence. RSV attaches to the host cell through the G protein, which then allows the host and viral plasma membranes to fuse, allowing virus passage into the host cell. The F protein also encourages multinucleated cells to clump together by fusing their plasma membranes, resulting in the syncytial for which the virus is called and allowing virus transmission from cell to cell (Cartee *et al.,* 2003).

Human respiratory syncytial virus (RSV) is the leading cause of infant morbidity from respiratory infections worldwide, and it is so infectious by the age of two that virtually every child is at risk of contracting it (Paeray, 2004). Infection with the respiratory syncytial virus (RSV) causes serious bronchiolitis and pneumonia in children. The virus is responsible for about half of all pneumonia cases and up to 90% of all bronchiolitis cases in infants. It can also put children at risk for developing asthma, which is the most common chronic disease in children (Aliyu, 2009).

Near contact with infected individuals or contact with contaminated surfaces or objects spreads the virus from respiratory secretions. Infection can happen when infectious materials come into contact with the mucous membranes of the eyes, mouth, or nose, as well as by inhalation of droplet nuclei formed by sneezing or coughing. Fever, runny nose, cough, and sometimes wheezing are the most common symptoms (Welliver, 2003; Flores, 2008; Linder & Malani, 2017). Nearly all children become infected by the age of two, but the highest occurrence occurs between the ages of two and three months, when the drug used to protect maternal IgG transmitted to the fetus via the placenta are in lowest concentrations.

Seasonal outbreaks occur every year all over the world, but their onset, peak, and length differ from year to year (Jackson and Lemanske, 2010). Respiratory syncytial virus (RSV) epidemiology varies greatly depending on latitude and weather conditions. RSV production, for example, appears to be year-round at sites with consistently warm temperatures and high humidity, peaking in the summer and early autumn. Respiratory syncytial virus activity is highest in temperate climates during the winter and correlates with lower temperatures. RSV activity becomes virtually constant in areas where temperatures are colder during the year. Thus, both ambient temperature and absolute humidity influence RSV operation in communities, possibly representing meteorological combinations that enable RSV to be more stable in aerosols (Yusuf *et al.,* 2007).

Premature children, as well as those with chronic lung disease (e.g., bronchopulmonary dysplasia, cystic fibrosis, and interstitial lung diseases) or hemodynamically severe congenital heart disease, have a higher morbidity and mortality from RSV disease (Renato *et al.,* 2017). Since preterm infants skip the third trimester window when the placenta expresses Fc receptors that mediate the transfer of maternal IgG to the fetus in part or entirely, they are born with lower humoral defenses against infection and maternal IgG concentrations. T-cell–mediated responses are unsuccessful, which is intensified by the fact that T cells mature mainly during the third trimester of pregnancy (Law *et al.,* 2004).By reducing pulmonary functional reserve, distorting airway architecture, and fostering a proinflammatory environment, bronchopulmonary dysplasia or other chronic respiratory conditions increase the risk of serious infections. Age less than 12 weeks, a history of prematurity, male sex, crowding, lack of breastfeeding, congenital heart disease, and any immunodeficiency are all additional risk factors for serious disease. Nonetheless, when treating infants and children for bronchiolitis, doctors may ask about cigarette smoke exposure and offer caregivers smoking cessation advice (Law *et al.,* 2004).

The presence of large antibody titers, previous RSV infection does not confer long-term immunity, though higher titers can slow the disease's progression (Nodler *et al.,* 2009). As a result, re-infection is normal, may happen within the same viral season, and affects people of all ages. Because of the restricted immunologic defense, narrower airway size, and specific structural and functional features of the developing respiratory tract, the first episodes of infection usually occur during the first two years of life and are the most serious (e.g., lack of inter alveolar pores and channels and different innervations patterns),(Green *et al.,* 2018).

Respiratory syncytial virus (RSV) is spread by inhaling aerosols from infected people coughing or sneezing (Flores, 2008; Gould and Dray, 2009). Individual of any age may contract RSV again, but infections later in life are usually milder. Premature babies, young children with congenital heart or chronic lung disease, young children with compromised (weakened) immune systems as a result of a medical condition or medical care, adults with immune-compromised immune systems, and older adults, particularly those with underlying heart or lung disease, are among those most at risk for serious disease (Aliyu, 2009).

Respiratory syncytial virus (RSV) vaccines are being developed by researchers, but none are currently available. Palivizumab is a medication used to prevent serious RSV infection in babies and children who are at high risk (Anderson *et al.,* 2017).

MATERIALS AND METHODS

Sample Collection

A random sampling technique was used collect blood samples from children aged 0 to 60 months attending Barau Dikko Teaching Hospital Kaduna and Yusuf Dantsoho Memorial Hospital Kaduna, A total of 96 blood samples were collected from each hospital.

A 2 ml syringe was used to collect venous blood from children in the study. The participants' arms were covered in a soft tourniquet to make the veins more visible. Prior to the blood draw, the site was swapped with cotton wool dipped in methylated spirit and allowed to air dry. The samples were clearly labeled and placed in an ice box (Chessbrough, 2012).

Nasal swabs were also obtained using sterile nylon swabs in 1ml of viral transport medium (Ebrahim *et al.,* 2016). The blood and nasal swab samples were immediately transported to the Department of Microbiology, Kaduna State University, Nigeria, for further study in an ice box.

Detection of IgM Antibody against RSV using Enzyme Linked Immunosorbent Assay

The serum was collected into sterile dried plain specimen bottles and processed at -20°C after centrifugation at 1500 revolutions per minute for five minutes (1500 rpm/min for 5min) (Aliyu, 2009). The collected serum samples were tested for unique IgM antibody against RSV using enzyme-linked immunosorbent assay (ELISA) (Aliyu, 2009).

Samples were tested for unique IgM antibody against RSV using ELISA (RSV IgM ELISA DEMEDITEC,GmbH Germany). The manufacturer's instructions were followed to the letter. Using a sigma Diagnostic EIA multi well reader, the optical density (OD) values were read at 450nm. The kit's specificity and sensitivity is also 100 *%*.

Molecular Analysis

RNA Extraction

In a 1.5ml eppendorf tube, 200µL of Sample (nasal swab) was added, 400ul of Binding buffer (VB) was added, and the tube was vortexed for 5 seconds. It was also incubated at room temperature for 10 minutes. After adding 100µl of isopropanol and vortexing it for around 5 seconds, it was spun down for 10 seconds to remove any liquid sticking to the tube's walls and lid. The binding column was then inserted into the 2ml collection tube. The liquid was then/ moved to the binding column without getting damp on the lid. The lid was closed carefully and centrifuged for 1 minute at 8000 rpm. The binding column was then moved to a new 2 ml collection tube. The column was filled with 500µL of W1 buffer without making the sides wet, then the lid was closed and centrifuged for 1 minute at 8000 rpm. The binding column was moved to a 2ml collection tube after centrifugation, and 500µL of W2 buffer was applied without making the sides wet; the lid was centrifuged for 1 minute at 8000 rpm. It was spun down again at 13000 rpm for 2 minutes to fully extract the ethanol. The binding column was placed in a 1.5 ml collection tube, 50µL of Elution Buffer was added, and the column was allowed to permeate for 5 minutes. It was then eluted for 2 minutes by spinning at 8000 rpm. The eluted RNA solution can be used right away or kept at -70^{o}C for later used.

Polymerase Chain Reaction-Reverse Transcription (CDNA Synthesis)

The CDNA synthesis PCR was carried in a total volume of 20µl reaction with 15µl of the extracted RNA, 3µl deionized water (molecular grade distilled water) and 1µl of each G1 and G2 primers and the CDNA synthesis was carried out at 48^{0}c for one hour then 94^{0}c for 5 minutes (Nikfar *et al*., 2012).

After the CDNA synthesis, first round PCR was performed with the forward primer (G1-CCA TTC TGG CAA TGA TAA TCTC) and the reverse primer (G2-GTT TTT TGT TTG GTA TTC TTT TGC GA) to target 326bp fragment. It was carried out in a total volume of 20µl using hotstat PCR premix with 15µl of deionized water, 1µl of forward primer, lul of reverse primer and 3µl of CDNA. The PCR was carried out under the following conditions, 94^{0}c for 5 minute 35 cycles of 94^{0}c for 30 seconds, 50^{0}c for 30 seconds and 72^{0}c for 30 seconds, with a final extension of 72^{0}c for 10 minute and a 4^{0}c keep. Second round PCR was then carried out by adding 2µl of first round PCR, lul each primer (G3 and G4) and 17µl of deionized water, the amplicon conditions were as follows 94^{0}c for 5 minutes,35 cycles of 94^{0}cfor 30seconds, 55^{0}c for 30 seconds and a final extension at 72^{0}c for 5 minutes and 4^{0}c hold.

Gel Electrophoresis

Electrophoresis on a 2.0*%* agarose gel with 100bp^{+}molecular marker was used to examine the PCR (100bp ladder). Until UV visualization, the gels were stained with ethidium bromide (0.5g/ml) (Fall *et al.,* 2016).

RESULTS

In assessing the overall prevalence of respiratory syncytial virus (RSV), a total of 192 of both blood and nasal samples were collected, out of which 54/192 (56.0%) tested positive for all the samples analyzed from both hospitals (Barau Dikko Teaching Hospital and Yusuf Dantsoho Memorial Hospital). The percentage prevalence of positive samples recorded for Barau Dikko Teaching hospital was 25/96 (26.0%), while that of Yusuf Dantsoho Memorial hospital was 29/96 (30.0%) respectively as shown in Figure 1.

Table 1 shows the distribution of RSV with regards to socio-demographic characteristics. Males had a higher prevalence 60/69 (31.0%) than females 50/96 (26.0%) but the result was not statistically significant (*p- value*=0.285). Infants under one month had the highest prevalence rate of 70/93 (75%), while 31-60 month infants had the least prevalence rate of 4/35 (11.0%). The difference was statistically significant (p-value=0.001).

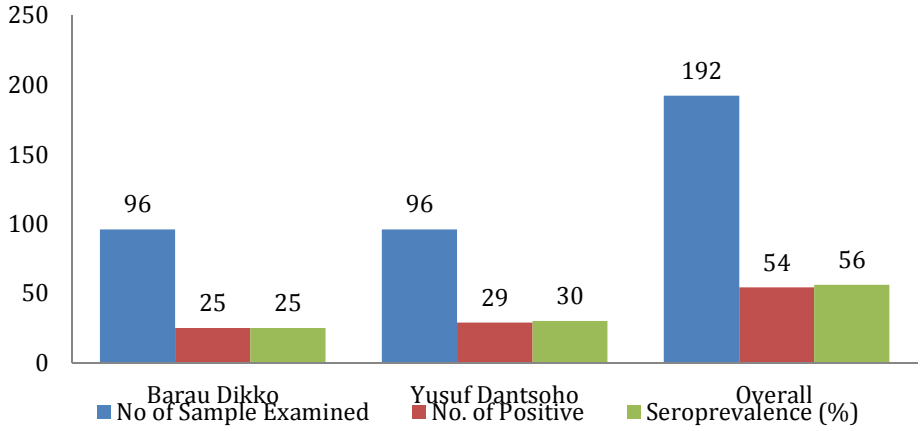

Figure 1: Seroprevalence of RSV among Children 0-60 Months Attending BDTH and YDH

Table 1: Prevalence of Respiratory Syncytial Virus in relation to Socio-Demographic Characteristics.

Demography data	Number tested	Seropositive (%)	χ^2	p-value
Age				
Under 1 month	93	70(75.0)	42.400	0.001**
1-30month	64	36(56.0)		
31-60month	35	4(11.0)		
Sex				
Male	96	60(62.5)	2.129	0.285
Female	96	50(52.1)		

Variable with ** are statistically significant at p<0.05

The molecular characteristic of RSV-G gene was confirmed by PCR and agarose gel electrophoresis (Figure 1**).** Bands show 326bp amplicons of rRNA primer targeting the RSV-G templates V3 hyper-variable region.

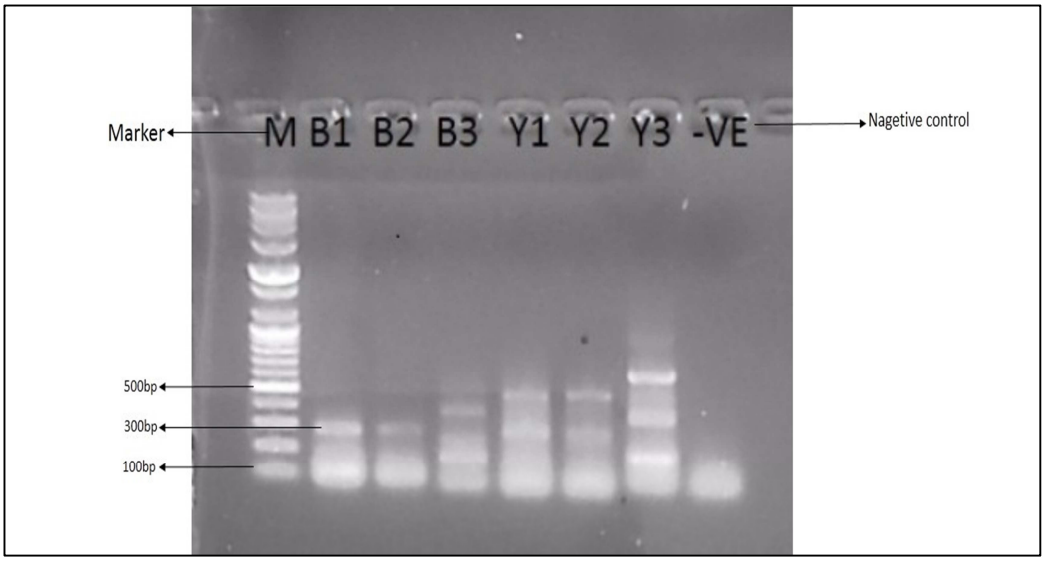

Plate 1. The Agarose gel electrophoregram of RSV G-gene where G-gene was detected in B1 and B2 with base pair 326bp.
KEY: BT- Barau Dikko, Y- Yusuf Dantsoho, M- Molecular maker (100bp), N- Negative control

DISCUSSION

The respiratory syncytial virus (RSV) is one the leading viral cause of death in children aged 0 to 60 months, and it is becoming a more common cause of morbidity and mortality in transplant patients and the elderly (Andrea and Jose, 2018). This study adds to the body of information about the burden of RSV infection among children aged 0 to 60 months who attend Barau Dikko Teaching Hospital and Yusuf

Dantsoho Memorial Hospital in Kaduna state, as well as the risk factors for infection.

In this study, out of the total of 192 samples collected, 110 were seropositive for RSV IgM antibody, giving an overall prevalence of 56*%*. This result present a lower prevalence compared to that of Aliyu (2009) who recorded 93.1%, prevalence rate in Zaria, Kaduna State, and Faneye *et al.* (2014), who reported an 85.7 *%* prevalence rate in North Central Nigeria. The prevalence was higher than the value reported in the findings of Rungnapan *et al.* (2013), who reported a prevalence rate of 28.1 *%* in the Philippines, and Lee *et al.,* (2007), who reported a prevalence rate of 23.1 *%* in Brazil, was against the value reported in the findings of Rungnapan *et al.* (2013), who reported a prevalence rate of 23.1 *%* in Brazil. Geographical location, time and season of collection, and method of detection used may all be factors in the prevalence rate's variation. Previous research has shown that maternally acquired antibodies can protect against RSV infection in infants (Bhattarakosol *et al.,* 2014). While high antibody titres have been shown to significantly reduce the incidence of RSV hospitalizations and disease severity, the presence of RSV antibodies alone is not enough to protect against re-infection (Collins *et al.,* 2014)

Males had a positive predominance over female but the difference was not statistically significant. The finding of Faneye *et al.*(2014), reveals that there were a higher percentage of male RSV positive cases 91.6% than female RSV positive cases 75.5%. Also the result of (Aliyu, 2009) reveals the findings of similar research with females 94.4% outnumbering males 92.1%. Regardless of gender, every infant is at risk of an infection. However, the demographic has no impact on the RSV prevalence rate.

This study also showed that as age increases i.e. 31-60 months the prevalence decreases showing a statistical significance. Higher prevalence was shown in younger children less than one month with a statistical relationship, there may be due to the maternal declining of antibodies acquired during placental transfer.

PCR was performed on 10 % of the ELISA positive samples. BDTH and YDS samples were selected at random, and two of them tested positive for PCR. In two samples from the Barau Dikko teaching hospital, the RSV-G gene was found.

This result was similar with the findings of Khalil *et al.* (2012) and Aya *et al.* (2014) whom also detected RSV-G gene in positive cases in Sudanese and Egyptian children respectively. More than 100 children presented the criteria for inclusion in the study. Samples from the first 100 patients were collected due to the limit established by processing and storage of samples. We did not check out all the respiratory viral agents in our patients. Accurate differentiation between viral and bacterial pneumonia is not possible according to clinical manifestation, some of the cases with negative PCR for RSV may have bacterial pneumonia. Antimicrobials administered children were not excluded from the studies. Children over the age of 60 months of both sexes who do not have respiratory symptoms were excluded.

CONCLUSION

In children aged 0 to 60 months, respiratory syncytial virus is one of the most common causes of respiratory viral infection. The virus was found to be prevalent in (56.0%) of children in the study. RSV infection was statistically related to age. In two of the samples examined, the RSV- G gene was detected.

Conflict of Interest/Acknowledgement/Funding

Acknowledgement

We appreciate Ministry of Health, Kaduna State for permitting us to use the hospital for sample collection. We wish to also thank the laboratory scientist of DNA labs, Kaduna State and laboratory scientists of Barau Dikko Teaching Hospitals and Yusuf Dantsoho Memorial Hospital who assisted in sample collection. We also appreciate the support of various volunteers who willingly participated in the research.

Conflict of Interest

The authors declare no conflicting interest regarding the publication of this paper.

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