Molecular Epidemiology and Disease Severity of Respiratory Syncytial Virus in Relation to Other Potential Pathogens in Children Hospitalized With Acute Respiratory Infection in Jordan

Nasser M. Kaplan,1,2 Winifred Dove,2* Sawsan A. Abd-Eldayem,1 Ahmad F. Abu-Zeid,3 Hiyam E. Shamoon,3 and C. Anthony Hart2
1Department of Microbiology, King Hussein Medical Centre, Amman, Jordan
2Department of Medical Microbiology, University of Liverpool, Liverpool, United Kingdom
3Department of Paediatrics, King Hussein Medical Centre, Amman, Jordan

Human respiratory syncytial virus (HRSV) is the major viral cause of acute lower respiratory tract infections in children. Few data about the molecular epidemiology of respiratory syncytial virus in developing countries, such as Jordan, are available. The frequency and severity of infections caused by HRSV were assessed in hospitalized Jordanian children <5 years of age compared with other potential etiological agents. Overall a potential pathogen was detected in 78% (254/326) of the children. HRSV was detected in 43% (140/326) of the nasopharyngeal aspirates. HRSV was found more frequently during the winter (January/February), being less frequent or negligible by spring (March/April). Analysis of 135 HRSV-positive strains using restriction fragment length polymorphism showed that 94 (70%) belonged to subgroup A, and 41 (30%) to subgroup B. There were also two cases of mixed genotypic infection. Only four of the six previously described N genotypes were detected with NP4 predominating. There were no associations between subgroup or N-genogroup and disease severity. HRSV was significantly associated with more severe acute respiratory infection and the median age of children with HRSV was lower than for those without. Next in order of frequency were adenovirus (116/312: 37%), human bocavirus (57/312: 18%), rhinovirus (36/325: 11%), Chlamydia spp. (14/312: 4.5%), human metapneumovirus (8/326: 2.5%), human coronavirus NL63 (4/325: 1.2%), and influenza A virus (2/323: 0.6%). Influenza B; parainfluenza viruses 1–4, human coronavirus HKU1 and Mycoplasma pneumoniae were not detected. J. Med. Virol. 80:168–174, 2008.

KEY WORDS: acute respiratory infections; respiratory syncytial virus; molecular epidemiology; disease severity; children; Jordan

INTRODUCTION

Acute respiratory infection is the major cause of death in children <5 years of age, and occurs predominantly in developing countries [Bryce et al., 2005]. Human respiratory syncytial virus (HRSV) is the leading viral cause of acute respiratory infection in infants and young children in terms of prevalence and effect [Shay et al., 2001]. HRSV causes substantial annual winter epidemics in temperate climates, representing a major cause of pediatric hospitalizations and a serious economic burden [Viegas et al., 2004]. HRSV can cause re-infections throughout the child’s life and it can infect infants in the presence of maternal antibodies. This could be due to either an inadequate immune response or to the extensive genetic variability of the virus. Although antigenic variation is not essential for reinfection, growing evidence suggests that it may contribute to re-infections by immune evasion [Viegas and Mis-tchenko, 2005]. It has also been reported that HRSV-specific T-cell responses do not provide protection.
against reinfection, and reinfection does not boost HRSV-specific T-cell proliferation [Bont et al., 2002].

HRSV is an enveloped RNA virus that is classified within the Pneumovirus genus as a member of the family Paramyxoviridae [Collins et al., 2001]. The virus has a non-segmented, single-stranded, negative-sense genome. Its genome encodes 10 proteins, including two major surface glycoproteins (G and F), two matrix proteins (M1 and M2), a small hydrophobic protein (SH), and three nucleocapsid associated proteins (N, P, and L) [Collins et al., 1984]. The two antigenic glycoproteins G and F are responsible for host cell attachment and viral entry by membrane fusion, respectively [Palomo et al., 2000]. HRSV is divided into two major antigenic subgroups, A and B, on the basis of reactivity with monoclonal antibodies against the major structural glycoproteins G and F [Mufson et al., 1985]. However, these two subgroups can be further subdivided into genotypes by restriction analysis and nucleotide sequence variability [Sullender et al., 1993; Peret et al., 1998]. The N (nucleoprotein) gene is relatively well conserved between virus isolates, but the G gene shows much greater variability. The variation in these genes has been used to define HRSV typing schemes as N gene differences resulting in the NP1-NP6 genotypes.

The purpose of the present study was to examine the molecular epidemiology of HRSV in Jordan. We also compared the disease severity of HRSV subgroups A and B and their associated genotypes in hospitalized Jordanian children set in the context of other potential respiratory pathogens.

MATERIALS AND METHODS

Study Design

This prospective cross-sectional study was conducted over six consecutive months from December 2003 to May 2004. Children younger than 5 years of age with acute respiratory infection admitted to the pediatric wards of King Hussein Medical Centre and Queen Alia Hospital, Amman, Jordan were enrolled in the study irrespective of the severity of their illness. King Hussein Medical Centre, a tertiary referral hospital, and Queen Alia Hospital, a district general hospital, provide hospital pediatric care for Amman, the capital city of Jordan, and its surroundings. The study was approved by the medical research ethical committee of the King Hussein Medical Centre, Amman, Jordan and signed informed consent was obtained from each of the children’s parents or legal guardians for participation in the study.

The clinical diagnosis of acute respiratory infection and assessment of its severity was made by using the World Health Organization standard protocol for acute respiratory infection based on the presence of cough, tachypnoea, chest indrawing, and wheezing for <7 days duration [Pio, 2003]. Severe disease was defined as present in children with a respiratory rate >60/min and chest indrawing. Oxygen saturation (pO2) was measured by using pulse oximetry (Nelcor Puritan Bennett NPB-195, UK) and a pO2 <85% used as the cut-off for giving supplementary oxygen. A standardized questionnaire containing clinical, socio-demographic, therapeutic, and outcome data was completed for each patient.

Nasopharyngeal aspirates were collected by instilling 1 ml sterile phosphate-buffered saline through a sterile nasopharyngeal mucous extractor. The aspirates were frozen at −80°C until analyzed in the Department of Medical Microbiology, University of Liverpool, UK. The genome of HRSV was detected in nasopharyngeal aspirates by reverse transcription-polymerase chain reaction (RT-PCR) [Greensill et al., 2003]. HRSV-positive strains were classified into subgroup A and B by restriction fragment length polymorphism analysis [Cane and Pringle, 1992].

Nucleic Acids Extraction

Total RNA and DNA were extracted separately from nasopharyngeal aspirates by using the commercial RNeasy and QIAamp DNA Mini Kits (Qiagen, Crawley, West Sussex, UK) according to manufacturer’s instructions.

Reverse Transcription-Polymerase Chain Reaction for HRSV

The primers: N1 (5'-GGA ACA AGT TGT GGT TTA TGA ATA TGC-3') and N2 (5'-CTT CTG TCA AGT CTA GCA CAC TGT AGT-3') were used to amplify the nucleocapsid (N) gene between nucleotides 858 and 1,135 giving a 278-bp product [Cane and Pringle, 1991].

An aliquot of 10 μl of the extracted viral RNA serving as a template for cDNA synthesis was added to 40 μl of PCR mixture. The final 50 μl mastermix reaction contained 1× PCR buffer, 3 mM MgCl2, 5 mM dithiothreitol (dTT), 0.4 mM deoxyribonucleotide triphosphates (dNTPs), 0.4 μM of primers mixture containing equal volumes of the two primers at a concentration of 20 μM each, 20 units (U) of RNase inhibitor (RNasin), 25 U of murine leukemia virus reverse-transcriptase, and 2.5 U of Amplitaq gold DNA polymerase (Roche Diagnostics Ltd., Burgess Hill, UK).

The RT-PCR was performed in a Perkin Elmer GeneAmp 2400 thermal cycler (Norwalk, CT) according to the following program: 30 min reverse transcription cycle at 50°C, followed by 5 min reverse transcriptase inactivation and DNA polymerase activation cycle at 94°C, then forty PCR cycles (1-min denaturation at 94°C, 1 min of primer annealing at 55°C, and 1 min of primer extension at 72°C), and a final extension cycle of 72°C for 10 min.

Ten microliter volumes of each amplified DNA PCR product were separated by electrophoresis on a 2% (wt/vol) agarose-Tris-Borate-ethylenediamine tetracetic acid gel, stained with ethidium bromide, and visualized under ultraviolet light (Syngene Gel Documentation and Analysis System, Ingenius, Cambridge, UK).

Restriction Fragment Length Polymorphism Analysis of HRSV

Aliquots of 2 μl from HRSV positive amplified DNA were separately digested directly and without prior...
purification by a set of five restriction endonuclease enzymes namely \textit{HindIII}, \textit{PstI}, \textit{BglII}, \textit{RsaI}, \textit{HaeIII} (Roche). The restriction patterns were analyzed by electrophoresis on a 2\% (wt/vol) agarose-tris-borate-ethylene diamine tetracetic acid gel, stained with ethidium bromide, and visualized under ultraviolet light. HRSV amplicons were typed into six nucleoprotein (NP) genotypes on the basis of their restriction endonuclease digestion profiles [Cane and Pringle, 1992]. HRSV subgroup A included NP2, NP4, and NP5, while subgroup B included NP1, NP3, and NP6 genotypes.

Detection of Other Respiratory Pathogens
Detection of other respiratory pathogens was performed according to previously published protocols. RT-PCR was used for the detection of human metapneumovirus (HMPV) [Greensill et al., 2003], influenza A and B, and parainfluenza virus 1–4 [Templeton et al., 2004], human rhinovirus (HRV) and human coronaviruses NL63 and HKU1 [Choi et al., 2006; Sloots et al., 2006]. PCR was used to detect human bocavirus (HBoV) [Allander et al., 2005], adenoviruses, \textit{Chlamydia} spp., and \textit{Mycoplasma pneumoniae} [Courouci et al., 2000].

Statistical Analysis
Epi Info Version 3.3.2 (Centers for Disease Control, Atlanta, GA) was used for statistical analysis by applying Chi-Square and Student’s \(t\)-tests. \(P\) values <0.05 were considered statistically significant.

RESULTS
A total of 326 children (188, 58\% male) with a median age of 5 months were recruited, and 326 nasopharyngeal aspirates were collected and analyzed, however the volume of some nasopharyngeal aspirates was inadequate for both DNA and RNA extractions. Of the 326 children studied 15 (5\%) had some underlying condition which might have contributed to disease severity. Of these 13 had cardiopulmonary disease, predominantly congenital heart disease (but none had bronchopulmonary dysplasia), one was premature and one had immunodeficiency. In all 8 (53\%) of those with an underlying condition had severe disease. A total of 72/326 (22\%) patients had no pathogens detected by PCR (Table I), but 254 (78\%) had at least one potential respiratory pathogen detected which consisted of 140/326 (43\%) HRSV, 116/312 (37\%) adenoviruses, 57/312 (18\%) HBoV, 36/325 (11\%) rhinovirus, 14/325 (4.5\%) \textit{Chlamydia} spp., 8/326 (2.5\%) HMPV, 4/325 (1.2\%) human coronavirus NL63, and 2/323 (0.6\%) influenza A virus. Overall 106/326 (33\%) infants had mixed infections (Table II) and of these 83 were infected with 2 potential pathogens, 22 with 3, and 1 with 4 potential pathogens. A total of 67 HRSV co-infections (48\% of all HRSV infections) were detected; 30 with adenoviruses, 10 with HBoV, 10 with adenovirus and HBoV, 8 with rhinoviruses, 3 with \textit{Chlamydia} spp., 1 with rhinovirus and HBoV, 1 with \textit{Chlamydia} spp. and adenovirus, 1 with \textit{Chlamydia} spp. and HBoV, 1 with \textit{Chlamydia} spp. and rhinovirus, 1 with adenovirus and rhinovirus, and 1 with \textit{Chlamydia} spp., rhinovirus and HBoV. No HRSV/HMPV co-infections were detected. Influenza B, parainfluenza viruses 1–4, human coronavirus HKU1 and \textit{M. pneumoniae} were not detected in this study.

Restriction fragment length polymorphism analysis was performed for 98\% (137/140) of HRSV-positive strains; three weakly positive strains were not subjected to further subgrouping analysis. HRSV subgroup A was detected in 70\% (94/135) and subgroup B in 30\% (41/135) of the samples. There were two cases of mixed genotypic infections, one NP2/NP4 (A/A) and one NP4/NP3 (A/B). Genotyping analysis of HRSV strains showed that 63 (47\%) were NP4, 31 (23\%) NP2, 26 (19\%) NP1, and 15 (11\%) NP3. Each of the four genotypes co-circulated during the January–March period of the study (Table III).

All the children in the study had lower respiratory tract infections mainly bronchiolitis and bronchopneumonia and routine cultures of their blood and respiratory secretions had detected no potential bacterial or fungal pathogens. Although there were no deaths, three children were admitted to the Intensive Care Unit, one infected only with HRSV subgroup B (NP1), one

| Pathogen         | No. NPAs tested | No. positive (no. mixed infections) | % Positive |
|------------------|-----------------|------------------------------------|------------|
| HRSV             | 326             | 140 (67)                           | 43         |
| Adenovirus       | 312             | 116 (75)                           | 37         |
| HBoV             | 312             | 57 (61)                            | 18         |
| HRV              | 325             | 36 (26)                            | 11         |
| \textit{Chlamydia} spp. | 312             | 14 (13)                            | 4.5        |
| HMPV             | 326             | 8 (4)                              | 2.5        |
| HCoV NL63        | 325             | 4 (1)                              | 1.2        |
| Influenza A      | 323             | 2 (0)                              | 0.6        |
| Influenza B      | 326             | 0                                  | 0          |
| Parainfluenza 1–4 | 328             | 0                                  | 0          |
| HCoV HKU1        | 325             | 0                                  | 0          |
| \textit{M. pneumoniae} | 312             | 0                                  | 0          |
| None             | 326             | 72                                 | 22         |

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infected only with HBoV, and one who had congenital heart disease with no pathogens detected. A total of 139 (43%) children had severe, and 187 (57%) had mild-moderate acute respiratory infection. Significantly \((P < 0.0005)\) more HRSV-infected children had severe disease (100/140; 71%) compared to those uninfected with HRSV (39/186; 21%). The median age of HRSV-infected patients was 4.8 months (range 1–48 months) and 82 (59%) were male, compared with a median age of 6 months and 104 (56%) male patients in the HRSV-negative patients \((P < 0.05)\). Severe HRSV infections occurred in all age groups, however it was significantly more likely to occur in those under 6 months of age \((P < 0.01)\). Thus 62 (62%) in the 0–6 months age group, 30 (30%) in the 7–12 months group, and 8 (8%) in those older than 1 year had severe disease. HRSV subgroup A and B were associated with 66 and 33 cases of severe disease, respectively \((P = 0.2)\). There was no significant difference between the individual HRSV genotypes as potential causes of severe disease.

In the 100 patients with severe acute respiratory infection in whom HRSV was detected, it was the sole pathogen detected in 53 (53%) patients, however in the remaining 47 cases, it was found as a mixed infection with adenovirus (20 patients), HBoV and adenovirus (9 patients), HBoV (8 patients), Chlamydia spp. (3 patients), rhinovirus (2 patients), HBoV and rhinovirus (1 patient), Chlamydia spp. and HBoV (1 patient), Chlamydia spp. and adenovirus (1 patient), adenovirus and rhinovirus (1 patient), and Chlamydia spp., HBoV and rhinovirus (1 patient). There was no significant difference \((P > 0.5)\) in the prevalence of severe disease between those where HRSV was the sole pathogen (53/73; 73%) and those with HRSV and another potential pathogen (47/67; 70%). The median age of children with severe acute respiratory infection was 4 months for those infected only with HRSV, and 6 months for those co-infected with HRSV and other potential respiratory pathogens \((P < 0.05)\). In the 40 patients with mild-moderate acute respiratory infection in whom HRSV was detected, it was the only pathogen in 20 (50%) patients, however in the remaining 20 cases, it was found as a mixed infection with adenovirus (10 patients), rhinovirus (6 patients), HBoV (2 patients), adenovirus and HBoV (1 patient), and Chlamydia spp. and rhinovirus (1 patient). The median age of children with mild-moderate acute respiratory infection was 4 months for those infected only with HRSV, and 9.5 months for those co-infected with HRSV and other potential respiratory pathogens \((P = 0.05)\).

Of the 36 children infected with rhinovirus 24 (67%) had mild/moderate and 12 (33%) severe disease. The median age of those infected with rhinovirus was 4.5 months not significantly different from that of those infected with HRSV. Although 12 of the children infected with rhinovirus had severe disease, only one of these severe infections occurred in the absence of coinfection with any of the other eleven potential respiratory pathogens. Of the 24 children with mild/moderate disease 17 (71%) were co-infected with other pathogens. There was a similar prevalence of rhinovirus infection in January–April.

### TABLE II. Respiratory Pathogen Co-Infections

| Pathogen | HRSV | Adenovirus | HBoV | HRV | Chlamydia spp. | HMPV | H6VNL63 |
|----------|------|------------|------|-----|----------------|------|---------|
| HRSV     | —    | —          | —    | —   | —              | —    | —       |
| Adenovirus| 30   | —          | —    | —   | —              | —    | —       |
| HBoV     | 10   | 15         | —    | —   | —              | —    | —       |
| HRV      | 8    | 7          | 5    | —   | 1              | —    | —       |
| Chlamydia spp. | 3   | 1          | 1    | —   | 1              | 0    | —       |
| HMPV     | 0    | 1          | 0    | 0   | 0              | —    | —       |
| HCoV NL63 | 0   | 1          | 0    | 0   | 0              | —    | —       |

Twenty-two children had three potential co-pathogens. One child had four potential co-pathogens.

### TABLE III. Results of HRSV, Its Subgroups and NP Genotypes by Month of the Study

| Month       | NPAs Tested | HRSV Positive | Subgroup A | Genotype NP2 | Genotype NP4 | Subgroup B | Genotype NP4 | Genotype NP1 | Genotype NP3 |
|-------------|-------------|---------------|------------|--------------|--------------|------------|--------------|--------------|--------------|
| December 2003 | 11          | 2             | 1          | 0            | 1            | 1          | 0            | 1            |             |
| January 2004  | 103         | 43            | 29         | 6            | 23           | 10         | 5            | 5            |             |
| February 2004 | 118         | 71            | 48         | 18           | 30           | 22         | 16           | 6            |             |
| March 2004    | 63          | 21            | 14         | 7            | 7            | 7          | 5            | 2            |             |
| April 2004    | 27          | 3             | 2          | 0            | 2            | 1          | 0            | 1            |             |
| May 2004      | 4           | 0             | 0          | 0            | 0            | 0          | 0            | 0            |             |

| Total (%)     | 326         | 140 (43%)     | 94/135 (70%)| 31 (23%)    | 63 (47%)     | 41/135 (30%)| 26 (19%)    | 15 (11%)     |

aNasopharyngeal aspirates.

bThree HRSV-weakly positive strains were not subjected to subgroup analysis by Restriction fragment length polymorphism.

cOne HRSV-positive strain showed simultaneous mixed infection by subgroups A (NP4) and B (NP3).

dOne HRSV-positive strain showed simultaneous mixed infection by genotypes NP2 and NP4 of subgroup A.
DISCUSSION

This study has confirmed that HRSV is the most frequent cause of severe acute respiratory infection in young children in Jordan. The fact that a large proportion of HRSV infections (71%) were associated with more severe disease is not unexpected because only hospitalized patients were involved in this study. However significantly more hospitalized infants with severe respiratory disease than mild/moderate disease due to HRSV were detected compared to infection with the other potential pathogens.

Worldwide, two subgroups of HRSV circulate independently within human populations, with group A being the more prevalent [Peret et al., 2000]. In a 3-year study conducted in the USA [Walsh et al., 1997], group B viruses predominated for 2 years, while group A predominated in the third year. Information on the relative frequencies of subgroups A and B in the Middle East and Africa is scarce. In the present study, subgroup A was predominant (70% vs. 30%), as previously shown in Jordan [Bdour, 2001], and Yemen [Al-Sonboli et al., 2005]. This predominance of subgroup A HRSV is the most common pattern worldwide [Cane, 2001].

Only four of the six previously described N genotypes [Fletcher et al., 1997] were found among the HRSV strains. All four genotypes were identified during the January–March period of the study, with genotype NP4 predominating (Table II). However all four genotypes co-circulated at the same time. Some authors have concluded that infection with group A HRSV is associated with more severe disease [Peret et al., 2000] but others have found no such association [Fletcher et al., 1997; Walsh et al., 1997]. In the present study HRSV subgroup A infection was not associated with more severe disease than infection with subgroup B (Table III). The medical staffs were unaware of group and genotype results and thus potential bias leading to a differential misclassification of the disease severity is unlikely.

Although the study extended through only 6 months of the year, there was a demonstrable peak of acute respiratory infection due to HRSV during January and February, as shown in a previous study in Jordan [Al-Teum et al., 2006]. HRSV was less frequent or almost absent in March–May.

HRSV-infected children were significantly younger than HRSV-negative children, and severe HRSV infections most frequently (90%) affected children younger than 12 months of age in agreement with a previous report from Jordan [Meqdam and Nasrallah, 2000]. Although it has been shown previously that children infected with group A HRSV were significantly older than those infected with group B HRSV [Hall et al., 1990], there was no significant difference in the ages of these groups (P = 0.2) in the present study.

There were no significant differences between the different HRSV genotypes as potential causes of severe disease, so it was difficult to establish a relationship between genotypes and disease severity.

Overall 102 (31.2%) of the children had more than one potential pathogen detected in their nasopharyngeal aspirates. This is perhaps not surprising as the respiratory viral seasons often coincide [Choi et al., 2006], and increasing the range of potential pathogens sought (in this study 10 viral and 2 bacterial pathogens) will reveal more co-infections. In 68 children the co-pathogens were HRSV with one or more other pathogen. However in no case was a co-infection with HRSV and HMPV encountered. Such co-infection has previously been linked with more severe acute respiratory infection in some [Greensill et al., 2003; Semple et al., 2005; Foulongne et al., 2006] but not all studies [Maggi et al., 2003; Wilkesmann et al., 2006]. HMPV was less frequently detected (2.5% of cases) than in some other hospital based studies [Al-Sonboli et al., 2005; Semple et al., 2005]. However the peak prevalence of HMPV infections does seem to vary year by year and the HRSV and HMPV seasons are not always concurrent [Serafino et al., 2004; Choi et al., 2006].

After adenovirus the second most common HRSV co-pathogen was the newly described HBoV. The finding of HBoV in Jordanian children has already been reported [Kaplan et al., 2006]. It was infrequently found as sole pathogen which suggested that further studies were needed to assess its role as a respiratory pathogen. However recent report from Edinburgh, UK has demonstrated that it is an important respiratory pathogen in children and not found in children without acute respiratory infection [Manning et al., 2006].

Adenoviruses were detected in 116 of the 312 children (37%), a prevalence that is significantly higher than those reported recently from Germany (12.9%), Brazil (6%), and India (1.5%) [Grondahl et al., 1999; Maitreyi et al., 2000; Straliotto et al., 2002]. However only the German study used RT-PCR. The latter two employed immunofluoresence and viral culture, respectively. In 75 (65%) of the cases of adenovirus infection it was found together with another potential pathogen. The German study showed that in 5% of the cases when an agent was detected there was more than one potential pathogen and in most cases this was adenovirus with another pathogen [Grondahl et al., 1999]. It is unclear why we had such a high detection rate of adenovirus infection and it warrants further study.

Rhinoviruses were the fourth commonest respiratory pathogen detected (36.11%). This prevalence is double that (5.8%) described for lower respiratory tract infections in Korean children [Choi et al., 2006] but somewhat lower than that (44%) found recently in Australia [Arden et al., 2006]. Rhinovirus was present as a co-pathogen in 24 (67%) of Jordanian children whereas in Australia in almost one-third of the rhinovirus infections a co-pathogen was detected [Arden et al., 2006]. HRVs are well-recognized causes of upper respiratory tract infection. However in the Australian study it was detected in almost half the samples from patients with lower respiratory tract infection [Arden et al., 2006], again somewhere higher than the 11% prevalence in Jordanian children, Hayden [2004] has recently
reviewed the role of rhinoviruses in lower respiratory tract infection and found that they were responsible for between 12% and 24% of such infections in children. In one study it was present as a co-pathogen in almost 50% of infections [Papadopoulos et al., 2002].

Better therapies and prevention strategies are needed to decrease the burden of acute respiratory infection particularly that due to HRSV. Thus further molecular epidemiological studies over longer periods of time are warranted to better determine the role of the different HRSV genotypes in the epidemiology and the severity of disease and their inter-relationship with other respiratory pathogens. This could inform better therapeutic approaches and vaccine development.

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