

Viral agents causing lower respiratory tract infections in hospitalized children: evaluation of the Speed-Oligo® RSV assay for the detection of respiratory syncytial virus

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Abstract Respiratory syncytial virus (RSV) is the viral agent which is more frequently involved in lower respiratory tract infections (LRTIs) in infants under 1 year of age in developed countries. A new oligochromatographic assay, Speed-Oligo® RSV, was designed and optimized for the specific detection and identification of RSV subtypes A and B. The test was evaluated in 289 clinical samples from 169 hospitalized children using an immunochromatography (IC) test, virus isolation by culture, and an in-house real-time polymerase chain reaction (RT-PCR). Other viruses causing LRTIs were investigated by cell culture or PCR-based tests. Sixty-two patients were infected by RSV (36.7%). In addition, adenovirus, influenza B, parainfluenza 2, and human metapneumovirus were detected in rates ranging from 5 to 8%. A proportion of 10.1% of the patients had mixed infections. The sensitivity, specificity, and positive and negative predictive values were, respectively, 94.9, 99.4, 98.9, and 97.4% for Speed-Oligo® RSV, 92.9, 96.3,

92.9, and 96.3% for RT-PCR/RSV, and 58.4, 98.1, 93.3, and 82.6% for IC. Our rates of viral detection and co-infection were similar to those of previously reported series. Finally, we find that Speed-Oligo® RSV is a rapid and easy-to-perform technique for the detection of RSV and the identification of subtypes A and B.

Introduction

Bronchiolitis is the leading cause of hospitalization in infants less than 1 year of age in developed countries and is mainly due to respiratory syncytial virus (RSV) [1]. Although the mortality from RSV infection is only <0.1% in developed countries [2], this infection increases the risk of serious complications in infants with cardiovascular diseases, cystic fibrosis, or immunodeficiency, among other conditions [3–5]. Besides infants, the elderly and severely immunocompromised individuals are also at higher risk of serious RSV infections [6, 7].

RSV epidemics mainly occur in the winter in temperate climates. During its seasonal peaks, it is the most frequently isolated virus from children with acute lower respiratory tract infection (LRTI) [8, 9] and is associated with high rates of hospitalization and emergency department visits [10, 11]. RSV infection represents the etiological diagnosis of the majority of severe respiratory infections in children, and the detection of this virus is critical for controlling its nosocomial transmission, isolating the patients according to their RSV status, and designating nursing staff accordingly [12, 13]. RSV detection was also found to reduce unnecessary antibiotic treatments [14, 15] and the length of hospital stay [14].

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The early and sensitive detection of RSV is especially important when RSV and influenza (IV) epidemics coincide and hospitalization rates are at their highest [16]. In addition to RSV and IV, rhinovirus (RV), adenovirus (AV), parainfluenza virus (PIV), and the recently discovered human metapneumovirus (hMPV) have been frequently implicated in LRTI episodes [17]. Diagnostic methods used for viral detection include cell culture, immunofluorescence/antigen detection, and nucleic acid/polymerase chain reaction (PCR)-based tests [17].

Virus isolation in cell culture remains a useful approach for viral disease diagnosis, but the delay in obtaining results represents a drawback. Antigen detection by immunochromatography (IC) tests is an option for laboratories not equipped for molecular testing, but their sensitivity is low, especially in adult patients [18, 19]. Tests based on nucleic acid amplification are rapid and sensitive, and appear to be the most suitable approach, but their high cost has impeded their widespread adoption as the first-choice diagnostic technique [20]. RSV detection has been studied by traditional PCR [21, 22], PCR-ELISA (enzyme linked immunosorbent assay) [23–25], liquid arrays [26], and real-time PCR [27–29].

The objective of the present study was to evaluate a new commercial assay [Speed-Oligo® RSV (SpO-RSV), Vircell] for the detection of RSV in human clinical samples. In addition to RSV, other viral agents involved in LRTI were studied using cell culture (AV, IVA, IVB, PIV1, PIV2 and PIV3) or molecular methods [RT-PCR to hMPV (RT-PCR/hMPV) and an experimental oligochromatographic assay to hMPV (SpO-hMPV), similar in design to SpO-RSV].

SpO-RSV is a PCR-based method coupled to a dipstick device that enables a rapid and less labor-intensive detection of the amplification products, yielding results within a short overall turnaround time (≤ 3 h), including RNA extraction, reverse transcription, PCR, and strip hybridization. Over a 1-year period, both a nasal wash (NW) and an oropharyngeal swab (OPS) were obtained from all patients with LRTI admitted to the Pediatric Department of Torrecárdenas Hospital, a tertiary hospital in Almería, southern Spain. The results of the SpO-RSV assay were compared with those of IC testing, in-house RT-PCR to RSV (RT-PCR/RSV), and viral culture. The applicability of the assay for routine RSV testing is discussed.

Methods

Patients and microbiological specimens

The study included all 169 pediatric patients diagnosed with LRTI and admitted to Torrecárdenas Hospital from August 2008 through August 2009, collecting 289 specimens

(163 NWs and 126 OPSs). Parents were informed and consent was signed for every recruited patient. Briefly, one tube of an aspiration trap was connected to a vacuum source and the other to a suction catheter of appropriate size. Then, 2–3 ml of sterile physiological saline was instilled into one nostril, and the aspirate (NW) was collected. The same procedure was done in the other nostril, with the same aspiration trap. For the OPS, a swab from the posterior pharynx was placed in viral transport medium (Vircell, Spain and Biomedics, Spain) and both NW and OPS were rapidly transported to the laboratory and kept at 4°C for a maximum of 24 h. Then, an aliquot of each NW was tested by IC (TRU RSV[®], Meridian Bioscience), and the remaining NW portion and the OPS were frozen at –80°C until they were sent to Vircell Laboratories for virus isolation and molecular testing. The project was previously approved by the regional research committee.

Immunochromatography test (TRU RSV[®], Meridian Bioscience)

Briefly, 100 μ l of the NW were added to a tube containing 100 μ l of sample diluents, and the mixture was vortexed for 10 s. A test strip was added to the tube and secured in place. The results were read after 15 min of incubation at 20–25°C.

Virus isolation

In a preliminary step, sterile beds were added to the samples and then vortexed; for OPSs, the swabs were discarded after vortexing. Samples were decontaminated by adding a 10% antibiotic mixture (BioWhittaker Lonza, USA) and incubating for 1 h at 4°C. A volume of 200 μ l of sample was inoculated into shell-vial tubes of each of the following cell lines: two Hep-2, two MDCK, and one LLC-MK2 (Vircell, Spain). Shell-vials were centrifuged at 700 g for 45 min. After centrifugation, shell-vials were incubated at 37°C for 1 h before discarding the inoculums; 1 ml of maintenance medium (minimal essential medium [Biowhittaker Lonza, USA] without fetal bovine serum) was added to Hep-2 and the shell-vial of LLC-MK2, while 1 ml of special maintenance medium (minimal essential medium [Biowhittaker Lonza, USA] without fetal bovine serum and 2 μ l/ml of TCPK-treated trypsin [Worthington Biochemical, USA]) was added to MDCK shell-vials. The Hep-2 and MDCK shell-vials and the LLC-MK2 shell-vial were incubated for 48 h. They were stained in a direct immunofluorescence assay with the following monoclonal antibodies: one Hep-2 shell-vial for RSV, one Hep-2 shell-vial for adenovirus, one MDCK shell-vial for Influenza A, one MDCK shell-vial for Influenza B, and one LLC-MK2 shell-vial for parainfluenza 1, 2, and 3 (all monoclonal antibodies from Vircell, Spain). Stained shell vials were examined in a fluorescence microscope at 400 \times magnification.

Nucleic acid isolation

The QIAamp Viral RNA Mini spin protocol (Qiagen, Germany) was followed in order to extract nucleic acids from clinical specimens, using 140 μ l of nasopharyngeal washes or pharyngeal swabs in transport medium. Finally, the nucleic acids were eluted in 60 μ l of the elution buffer included in the kit and stored at -80°C until analysis by RT-PCR and SpO-RSV.

Sequence analysis

The Beacon Designer 7 program (Premier Biosoft, Palo Alto, CA) was used for primer and probe design during SpO-RSV development. Primers and probes were designed to discriminate between RSV subtypes A and B in a single PCR, using the polymerase L gene as the target. Different reference sequences were used for the detection of RSV subtypes A (accession numbers: M75730 and U35343) and B (accession numbers: AY353550.1 and AF013254.1).

cDNA synthesis

The isolated RNA was reverse-transcribed using the AffinityScript Multiple Temperature Reverse Transcriptase system (Stratagene, USA). Each reaction contained 5 μ l of extracted RNA, 2 μ l of $10\times$ AffinityScript RT buffer, 2 μ l of 100 mM DTT, 4 μ l of 2.5 mM dNTPs, 1 μ l of AffinityScript Multiple Temperature Reverse Transcriptase, and 1 μ l of 100 mM random hexamers (Fermentas, Lithuania) in a 20- μ l final volume. After mixing, the tubes were incubated at 42°C for 1 h and then, for enzyme inactivation, at 70°C for 15 min. The cDNA obtained during the reverse transcription process was used in the oligochromatographic assays and real-time PCR.

Oligochromatographic assay (Speed-Oligo[®] RSV)

SpO-RSV is a commercial PCR-based method coupled to a dipstick device that enables RSV subtype A and subtype B identification in respiratory samples. The PCR mix was supplied in a lyophilized format and contains an additional oligo pair for a 138-bp human β -globin gene fragment, used as the internal amplification control; this was co-amplified simultaneously with the target sequence for RSV, a fragment of the RNA polymerase (L) gene, to allow testing of the quality and absence of inhibitory substances in the sample and the detection of thermal cycler malfunction, incorrect PCR mixture, or poor DNA polymerase activity [30, 31]. The PCR was carried out in a final volume of 25 μ l (5 μ l of cDNA plus 20 μ l of PCR mix). The PCR was performed in a Labcycler instrument (Sensoquest, Germany) with the following program: 1 min at 92°C , 45

cycles of $92^{\circ}\text{C}/15\text{ s}+55^{\circ}\text{C}/15\text{ s}+72^{\circ}\text{C}/15\text{ s}$, and one cycle of $72^{\circ}\text{C}/1\text{ min}+95^{\circ}\text{C}/1\text{ min}$. PCR products were detected by means of the dipstick, following the kit instructions. To perform the hybridization, 5 μ l of denatured PCR product were diluted in 35 μ l of a running solution and placed in a thermal block set at 55°C . The amplification products were hybridized on a dipstick using two specific probes bound to colloidal gold and four probes immobilized onto the membrane. The running of the test is described elsewhere [32, 33]. The strip has two specific bands for RSV subtype A and RSV subtype B, and a third specific line (amplification control line) that appears when the human β globin gene of the sample is amplified. A control line at the top of the strip reacts with excess colloidal gold and monitors the flow of the liquid along the strip. The final reading was visually accomplished after 5 min of incubation. Reactivity was confirmed by the visualization of a red line (Fig. 1). The entire process lasted $<80\text{ min}$.

Oligochromatographic assay (SpO-hMPV)

SpO-hMPV is an experimental PCR-based method similar to the previously described SpO-RSV. A 147-bp fragment of the N gene was amplified in a final volume of 25 μ l (5 μ l of cDNA plus 20 μ l of PCR mix). The amplification was performed in a Labcycler instrument (Sensoquest, Germany) with the following program: 1 min at 95°C , 40 cycles of $95^{\circ}\text{C}/20\text{ s}+60^{\circ}\text{C}/20\text{ s}+72^{\circ}\text{C}/20\text{ s}$, and one cycle of $72^{\circ}\text{C}/1\text{ min}$.

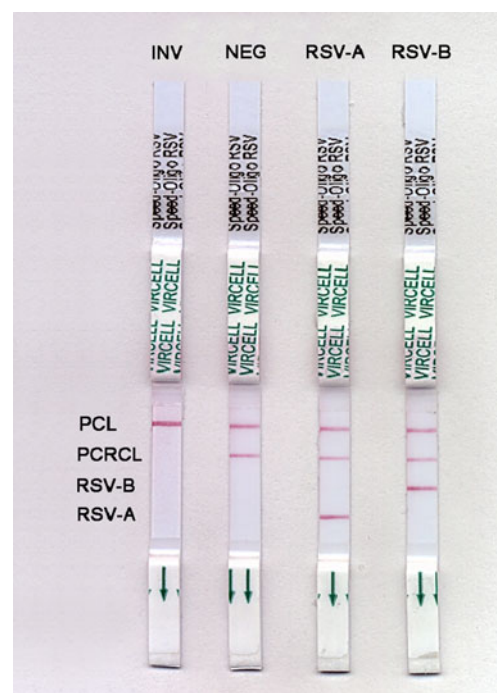


Fig. 1 Hybridization results of an invalid test (INV), negative test (NEG), and positive test (RSV-A, RSV-B). PCRCL: amplification control line; PCL: product control line

Real-time PCR (RT-PCR/RSV)

The N gene of RSV subtypes A and B was selected as the target for real-time amplification. Primers and probes recommended by Mlinaric-Galinovic et al. were used [34]. The specific probe for RSV subtype A and subtype B were labeled with HEX and FAM at the 5' end, respectively. Both probes were labeled with BHQ-1 quencher at the 3' end. Samples were assayed in a 25- μ l reaction mixture with 5 μ l of cDNA, using Brilliant II QPCR Master Mix (Stratagene, USA). All primers and probes were combined in a multiple PCR following van Elden et al. [35]. Amplification and detection were performed in a Stratagene Mx3005P real-time thermocycler with the following program: 10 min at 95°C to activate the Taq polymerase, and 45 cycles of 15 s at 95°C, 20 s at 60°C, and 20 s at 72°C. A positive and a negative control were included in each run.

Real-time PCR (RT-PCR/hMPV)

Primers recommended by Ebihara et al. [36] were used. A 142-bp fragment of the F gene was amplified. The PCR conditions were the same as the authors' recommended conditions using real-time mix with Kapa Sybr Fast (Kapa Biosystems). The amplification program was as follows: 3 min at 95°C, 40 cycles of 95°C/20 s+60°C/20 s+72°C/20 s, and a melting curve of 1 min at 95°C+30 s at 55°C and slope until 95°C with continuous reading to check the specificity of the amplified product.

Statistical methods

Comparisons between assays were done using the McNemar nonparametric test for paired proportions. A specimen was considered to be a true-positive when it was positive by culture or by both molecular tests (RT-PCR and SpO-RSV), and a patient with at least one such specimen was considered to be RSV-infected. All reported *p*-values are two-sided, and *p*<0.05 was considered to be significant. The Cohen's kappa

index was used to determine the concordance between the RT-PCR and SpO-RSV tests.

Results

A total of 289 specimens were tested. Both NW and OPS were available for 120 patients, NW alone for 43 patients, and OPS alone for six patients. The mean age of the 169 patients was 1.88 years (range, 14 days to 13 years).

Sixty-two patients (36.7%) had at least one positive specimen for RSV (57 RSV subtype A, three RSV subtype B, and two unknown subtype). All 289 specimens were assayed by SpO-RSV, RT-PCR/RSV, SpO-hMPV, RT-PCR/hMPV, and cell culture, while only 160 NWs were assayed by IC. The sensitivity, specificity, and positive and negative predictive values (in %) were 94.9, 99.4, 98.9, and 97.4, respectively, for SpO-RSV, and 92.9, 96.3, 92.9, and 96.3, respectively, for RT-PCR/RSV. Viral culture showed a sensitivity of 46.4% and a negative predictive value of 78.1%. IC showed a sensitivity of 58.4%, specificity of 98.1%, predictive positive value of 93.9%, and negative predictive value of 82.6% (Table 1). SpO-RSV and RT-PCR/RSV did not significantly differ in sensitivity (*p*=0.47) or specificity (*p*=0.077). Both PCR-based methods were significantly more sensitive than IC (*p*<0.0001) and viral culture (*p*<0.0001).

The performance of the two molecular techniques (SpO-RSV and RT-PCR/RSV) was very similar, and a very high concordance was found (kappa index 0.9224; SE=0.0588). Overall, 31.8% (92/289) of specimens were positive and 64.7% (187/289) were negative by both techniques, which only differed in the evaluation of 3.5% (10/289) of specimens, as reported in detail in Table 2.

For hMPV, 16 specimens (five OPS and 11 NW) corresponding to 13 patients (7.7%), had a positive result by both RT-PCR/hMPV and SpO-hMPV. Six patients were also positive for other viruses (three hMPV + RSV, one hMPV + RSV + IVB, one hMPV + IVB, and one hMPV + PIV2).

Table 1 Accuracy of Speed-Oligo® RSV (SpO-RSV), real-time polymerase chain reaction (RT-PCR), TRU RSV®, and viral culture*

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	TP	FP	TN	FN
SpO-RSV (<i>n</i> =289)	94.9 (90.6–99.2)	99.4 (98.4–1.0)	98.9 (96.9–1.0)	97.4 (95.1–99.6)	94	1	189	5
RT-PCR (<i>n</i> =289)	92.9 (87.8–97.9)	96.3 (93.6–98.9)	92.9 (87.8–97.9)	96.3 (93.6–98.9)	92	7	183	7
RSV IC (TRUE RSV ©) (<i>n</i> =160)	58.4 (45.2–71.7)	98.1 (95.5–1.0)	93.9 (85.8–1.0)	82.6 (76.1–89.2)	31	2	105	22
RSV culture (<i>n</i> =289)	46.4 (36.6–56.2)	–	–	78.1 (73.0–83.3)	46	0	190	53

PPV = positive predictive value; NPV = negative predictive value; TP = true-positives; FP = false-positives; TN = true-negatives; FN = false-negatives; *n* = no. of samples tested; values in parentheses are 95% confidence intervals

*Specimens were defined as positive if respiratory syncytial virus (RSV) was detected by viral isolation or by both polymerase chain reaction (PCR) assays

Table 2 Discrepant results between SpO-RSV and RT-PCR

	Specimen	RT-PCR	SpO-RSV	Culture	IC
Patients 1, 2, 3, 4, 5	NW	RSV A	Neg.	Neg.	Neg.
	OPS	Neg.	Neg.	Neg.	–
Patients 6, 7	NW	RSV A	RSV A	Neg.	Neg.
	OPS	RSV A	Neg.	Neg.	–
Patients 8, 9	NW	Neg.	RSV A	Pos.*	Neg.
	OPS	Neg.	Neg.	Pos.**	–
Patient 10	NW	Neg.	RSV B	Neg.	Neg.
	OPS	Neg.	Neg.	Neg.	–

*For both patients

**For one patient

Using cell culture, AV, IVB, PIV2, and RSV were isolated in 9 (5.3%), 11 (6.5%), 10 (5.9%), and 35 (20.7%) patients, respectively. Four patients had mixed viral cultures (RSV + AV in two cases, RSV + PIV1 in one case, and AV + PIV1 in one case). Overall, in 98 patients (58%), a viral etiology was found and ten of these patients (10.2%) had mixed infections. Among the 49 RSV-infected patients for whom both NW and OPS were available, the NW was positive in 45 cases and the OPS in 41 cases, although this difference did not reach statistical difference ($p=0.38$). When the analysis was done in regard to the RSV infection status of the patients, the sensitivity to detect RSV was 96.7% (60/62; 95% confidence interval [CI], 92.3–1.0) for SpO-RSV and 93.5% (58/62; 95%CI, 87.4–99.6) for RT-PCR/RSV.

Discussion

We evaluated a new commercial kit (Speed-Oligo® RSV) for RSV detection in two types of respiratory specimen (NW and OPS) from hospitalized pediatric patients with LRTI, using viral culture or positivity by both a real-time PCR protocol for RSV and the SpO-RSV as reference methods. We also evaluated the performance in NWs of TRU RSV[®], an IC test routinely used in our setting for RSV detection. Although numerous PCR methods have been developed to detect RSV in clinical samples [27–29], only a few can discriminate RSV subtypes A and B [35, 37, 38], which are distinguished by differences in the viral attachment (G) or nuclear (N) protein. It is still not clear whether the severity of infection differs between these subtypes. Some authors reported that subtype A infection was more severe [39], while others found no difference [2, 40]. No relationship has been observed between RSV epidemic patterns and circulating RSV subtypes, although it has been suggested that shifts in the predominating strain, even within the same subtype, may compromise a population's previously acquired immunity and favor the new strain [41]. RSV subtype percentages differ markedly among different countries, regions, and seasons, with a

predominance of either subtype A or B or the concurrent circulation of both [41, 42]. Thus, a study in Croatia found subtype B infections to be four-fold more common in two different outbreaks in Zagreb County [34], whereas a study in the United States found that 80% (602/753) of patients were infected with strain A, 18% with strain B, and 2% with both strains [40]. In our series, there was a very high predominance (95%) of subtype A infection, with only 5% of subtype B infection and no cases of co-infection.

In this study, we have proven viral infection in 98 patients (58%). RSV has been detected in 62 patients (36.7%) as a unique cause of infection (55 patients) or causing co-infection with other viruses (seven patients). Thus, RSV represented almost 2 out of 3 of our viral infections. Other viruses, such as hMPV, AV, PIV2, and IVB, had detection rates of 5–8%. The total rate of mixed infections was 10.1%. Although these data are similar to those of previously reported series [17], one limitation of this study is that RV was not investigated.

Molecular methods have proven to be more sensitive than the combination of conventional viral culture and shell vial culture in adults with various diseases [35, 43] and in infants, whose viral shedding is higher in comparison to adults [44, 45]. Improved detection of RSV is of particular importance in infants aged <1 year for whom RSV is the most common cause of bronchiolitis, as stated above. The use of RT-PCR in adults with respiratory RSV infection revealed a markedly higher number of positive cases in comparison to viral culture [27, 43]. The low sensitivity of viral culture, only 46.4% in the present study, has been attributed to low viral shedding and incorrect sampling methods [46]. It is crucial to transport samples on ice and process them within 2 h in order to improve the sensitivity of culture techniques [35], and some authors have underlined the need for the immediate culture of patient specimens to increasing cell culture sensitivity to detect RSV [47, 48]. The IC test used in our study (TRU RSV[®]) showed a rather low sensitivity (58.4%), but the specificity (98.1%) and PPV (93.9%) values were high, despite testing many of the specimens outside the RSV season, when the low prevalence

may decrease the specificity of this type of antigen detection test [49]. Rapid antigen detection assays have shown even lower sensitivity with respect to RT-PCR tests [50, 51]. The false-negative results by IC may be attributable to the small amount of viral particles in the sample [51].

In our study, the sensitivity values were similar for SpO-RSV and RT-PCR/RSV and in the same range as that previously reported for other PCR-based methods [34, 35], and the specificity was also very high for both SpO-RSV (99.4%) and RT-PCR/RSV (96.3%). In addition, SpO-RSV includes a PCR amplification internal control (human β -globin gene), since a false-negative result can result from the presence in the sample of PCR inhibitors not removed by the extraction process. No inhibition was observed in any of the samples tested by SpO-RSV.

This study also compared the efficacy of nasopharyngeal and oropharyngeal sampling, which are both widely used. The authors have reported that nasopharyngeal washes are superior to nasopharyngeal swabs for IV, RV, and coronavirus infections. Meerhoff et al. used RT-PCR and obtained 75% sensitivity in nasal swabs versus 97% in nasopharyngeal aspirates [52]. However, similar sensitivities have been obtained for RSV detection using different types of sample from pediatric [53] and adult populations [54]. In the present study, more positive results were obtained in NWs than in OPSs (45 vs. 41), but the difference was not significant. Maximal sensitivity values were achieved when both NW and OPS could be tested (96.7% for SpO-RSV and 93.5% for RT-PCR/RSV), as previously noted [54]. Further research is warranted in order to confirm that the use of both types of specimen increases the test sensitivity.

In conclusion, RSV is the viral agent which is more frequently involved in LRTI and, thus, rapid and accurate tests to detect this virus are vital not only to improve the etiological diagnosis of LRTI, but also to support procedures to reduce nosocomial infection among hospitalized children. Furthermore, the ability to discriminate RSV subtype discrimination can improve our understanding of RSV antigenic variability and facilitate future vaccine development. In the present study, the IC technique demonstrated a low sensitivity but very high specificity and positive predictive value, while PCR-based methods showed high sensitivity and specificity. In the hospital setting, the most cost-effective option for RSV diagnosis is IC methods. However, especially during the RSV season, when the prevalence is high, negative specimens should be re-tested with more sensitive PCR-based methods. According to our results, the Speed-Oligo[®] RSV is a rapid, easy to perform, and valid technique for this purpose.

Conflict of interest The authors declare that they have no conflict of interest.

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