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A sensitive real-time PCR for detection and subgrouping of human respiratory syncytial virus

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Improved diagnostic tools for rapid detection, quantitation, and subgrouping of human respiratory syncytial virus (RSV) are needed to aid the development and evaluation of novel intervention strategies. A quantitative real-time RT-PCR using specific locked nucleic acid (LNA) probes was developed to identify RSV and to distinguish RSV subgroups A and B (RSV LNA assay). RSV subgroup diversity and the relationship between viral load and disease severity in confirmed RSV infections were also explored. 264 archived respiratory specimens from pediatric patients were tested in parallel using the commercial multiplex Seeplex™ RV detection kit (Seegene) and the novel RSV LNA assay. The LNA assay demonstrated a significantly higher sensitivity than Seeplex, improving overall detection rates from 24% (64/264) to 32% (84/264). Detection limits of 9.0×10^1 and 6.0×10^2 copies/mL were observed for RSV A and B, respectively. RSV A was detected in 53/84 (63%) cases, and 31/84 (37%) were positive for RSV B. This novel method offers a rapid, quantitative, highly specific and sensitive approach to laboratory diagnosis of RSV.

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1. Introduction

Human respiratory syncytial virus (RSV) is a *Pneumovirus* and a member of the subfamily of *Pneumovirinae* within the family of *Paramyxoviridae*. Based on differences in the antigenicity and nucleotide sequences analysis, RSV strains have been classified in two major antigenic subgroups, denoted RSV A and RSV B (Sullender, 2000). RSV is a major cause of acute respiratory tract infections in children worldwide (Nair et al., 2010; Weber et al., 1998), and is responsible for a large proportion of hospital admissions during infancy and early childhood (Mejias et al., 2005). No vaccine is available, and treatment is limited to supportive care (Empey et al., 2010). RSV is also a frequent cause of nosocomial infections, and as such may significantly impact the duration of stay in hospital and healthcare costs during childhood (Macartney et al., 2000). Data on epidemiology and molecular virological characteristics of RSV isolates circulating in Southeast Asia are limited.

Diagnosis of RSV infection is typically performed on respiratory samples using virus culture, (rapid) antigen detection tests, or molecular assays (Henrickson, 2004). Real-time reverse transcriptase PCR (RT-PCR) provides a rapid and sensitive tool for detection of RSV compared to conventional techniques and enables (semi-)quantitation of viral load (Borg et al., 2003; Dewhurst-Maridor et al., 2004; do Nascimento et al., 2008; Empey et al., 2010; Falsey et al., 2003, 2002; Henrickson, 2004; Houben et al., 2010; Hu et al., 2003; Kuypers et al., 2004; Mentel et al., 2003; Perkins et al., 2005; van de Pol et al., 2006, 2010; van Elden et al., 2003). The locked nucleic acid (LNA) technology increases thermal stability of probe-target interaction and improves discrimination of different nucleic acid targets (Reynisson et al., 2006). The N gene is the most conserved RSV gene across all known genetic clades, and is also expressed most abundantly during viral replication (David and Knipe, 2001; Johnson and Collins, 1989). For these reasons, the N gene was chosen as the target of a novel real-time RT-PCR diagnostic tool, to provide quantitative viral load analysis and discrimination between RSV A and B within a single assay. The quantitative assay was compared to a commercial conventional multiplex PCR method (Seeplex™ RV detection kit, Seegene, Inc., Seoul, Korea) (Kim et al., 2009; Roh et al., 2008) using 264

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respiratory samples from a study (Do et al., 2011) on acute respiratory infection in children at the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. Here, the development and validation of this assay is reported, as well as initial observations regarding subgroup diversity within Vietnamese RSV strains, and relationships between viral load and disease severity in Vietnamese patients.

2. Methods

2.1. Clinical specimens

To develop and evaluate this novel RSV diagnostic, 264 respiratory samples, collected and stored as part of a study on acute respiratory infection among 309 children at the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam were used (Do et al., 2011). Specimens were kept at 4 °C for a maximum of 24 h and then aliquoted and stored at –80 °C until further processing. The samples comprised 240 nasopharyngeal aspirates, 23 throat swabs, and 1 nasal swab collected in viral transport medium, and represented cases admitted to the pediatric intensive care unit (ICU) (61/264, 23%) as well as the pediatric respiratory ward (203/264, 77%). One hundred and forty-one (53%) samples were from patients less than 2 years old, 113 (42%) from 2 to 5 years old, 11 (4%) over 5 years old.

All samples were analyzed in parallel by the commercial multiplex Seeplex™ RV detection kit (Seegene, Inc., Seoul, Korea) according to the manufacturer's instructions, to determine the presence of 12 respiratory viruses: human RSV subgroups A and B (RSV A, RSV B); influenza virus A (InfV A); influenza virus B (InfV B); human coronaviruses (229E, OC43), human metapneumovirus (hMPV), parainfluenza virus 1, 2 and 3 (PIV1, 2, 3), human rhinovirus (hRV A) and adenovirus (AdV) (Kim et al., 2009; Roh et al., 2008) and by the newly developed RSV LNA real-time RT-PCR.

2.2. RNA extraction and cDNA synthesis

RNA was extracted from 100 µL of specimen and eluted in 60 µL on the Easy MAG 2.0 system (bioMérieux, Marcy l'Étoile, France). An internal RNA-virus control (equine arteritis virus [EAV]) was added to each sample prior to extraction at a standard concentration yielding Ct values of 30–35 as previously described (Scheltinga et al., 2005). RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad (CA), USA) and random hexamers (Roche, Mannheim, Germany). Each 20 µL reaction mixture contained 5 µL extracted RNA, 1X RT-buffer (Invitrogen), 0.5 mM of each dNTP (Roche), 2 ng random hexamer, 10 mM DTT (Invitrogen), 1 U of RNase inhibitor and 2 U RT Superscript III. cDNA synthesis was performed using an Eppendorf Master thermocycler gradient system (PerkinElmer Corporation, Foster City (CA), USA) under the following conditions: 10 min at 25 °C, 60 min at 50 °C and 15 min at 75 °C.

2.3. Assay development

The assay was designed to detect all known RSV strains and to differentiate RSV subgroups A and B. Primers (MTH1 and MTH2B) were selected using Primer Express software v2.0 (Applied Biosystems Inc., Foster city (CA), USA) and a dataset of 45N gene sequences available from the NCBI database (Table 1) to amplify 84 bp for both RSV A and RSV B. RSV subgroups A and B differ by 5 nucleotides within the highly conserved region of the N gene; two LNA probes, RSV A and RSV B (Sigma Proligo, Singapore), were designed to include three LNA residues at these sites (+T, +A and +T for RSV A; +C, +G and +A for RSV B). The choice of reporters and compatible quenchers was based on a Bio-Rad guideline (Biorade, 2006). Primers and probes were analyzed for

false priming sites, hybridizations, and secondary structures at <http://lnatools.com/hybridization>. Plasmids containing the amplicons from RSV A and RSV B clinical strains were constructed for use as positive controls and generation of standard curves for quantitation. Briefly, target cDNAs amplified via RT-PCR from RSV A and RSV B-positive clinical specimens as identified by Seeplex™ RV detection kit, were cloned into pCRII®-TOPO® vector and transformed into the TOPO-10 bacterial strain using the TOPA TA cloning kit (Invitrogen). Clones were screened by PCR using M13 primers, and confirmed by sequencing (CEQ DTCs Quick Start Kit, CEQ 8000 Capillary Sequencer, Beckman Coulter, Inc., Fullerton (CA), USA). Quantitation of the plasmid preparations (ng/µl) was conducted using PicoGreen dsDNA Quantitation Reagent (Invitrogen) in duplicate, and the corresponding copy number of target cDNA in the plasmid was calculated (copies/mL). Serial 10-fold dilutions were made for analysis of analytical sensitivity and used as standard curves for viral load quantitation. Viral load was expressed as copies per mL of sample, A Ct-value of 45 was chosen as a cut-off value for standard positivity.

2.4. Assay protocol

The RSV LNA assay was performed on a DNA Engine Peltier Thermocycler and Chromo 4 Real-time PCR system detector (Bio-Rad, Hercules, USA), in a total volume of 25 µl containing 5 ul template cDNA, 0.05 U Hotstart Taq (Invitrogen), 1× PCR Buffer, 5 mM MgCl₂, 0.2 mM of each dNTP (Roche), 0.4 µM of each primer (RSV and EAV), 0.2 M of each probe for RSV, and 0.12 µM of EAV probe (Table 1). The PCR employed the following thermal cycler settings: 15 min at 95 °C, followed by 45 cycles of 30 s at 95 °C and 1 min at 45 °C. Negative controls and standards were included in every assay run. The number of RSV RNA transcripts (copies/mL) of clinical samples was determined based on the standard curve. A Ct-value of 45 was chosen as the cut-off value for sample positivity. All clinical samples required a Ct value of 30 to 35 for the EAV internal control to be considered valid.

Potential interference between primers/probes for target (RSV) and internal control (EAV) was assessed by running serial dilutions of two clinical samples with confirmed high viral loads of RSV A and RSV B (data not shown).

2.5. Assay performance assessment

The analytical limit of detection was determined using six 10-fold dilutions of control plasmids from 9.0×10^1 to 9.0×10^6 copies/mL for RSV A and 6.0×10^2 to 6.0×10^7 copies/mL for RSV B. The viral load in clinical samples was assessed by running them in parallel with quantitative standards. Since the concentration of target cDNA in plasmid controls was known, the corresponding concentration of cDNA in the clinical sample could be estimated. Samples that tested positive by RSV LNA but not by Seeplex were further confirmed by a seminested RT-PCR targeted at the second hypervariable region of the RSV G gene as described previously (Parveen et al., 2006). Specificity of the assay was assessed by presence of non-RSV viruses within the sample set (as determined by Seeplex). Specificity was further assessed by attempted amplification of culture supernatants of selected human seasonal influenza isolates [A/Sydney/5/97(H3N2), A/New Caledonia/20/99(H1N1), B/Yamanashi/166/98].

2.6. Statistical analysis

Statistical comparisons between Seeplex RT-PCR and the RSV-LNA assay were performed using McNemar's test. Assay variability was quantified by the standard deviation (SD) and coefficient of variation (CV). Ct-values (of five 10-fold dilutions of control

Table 1
Nucleotides sequences of primers and probes. LNA residues are shown in upper case and with a (+) sign in front.

Primers/probes name	Target	Nucleotide sequences
MTH1 (forward)	RSV N gene ^a	GGATTCTACCATATATTGA
MTH2B (reverse)		GAAGTKAGGAAATTGAGT
probe A0108 LNA (RSV A)		HEX-5'-ca + Aaagc + At + Cat + Ta + Tt + Atc + Ttt-BHQ1
probe B0108 LNA (RSV B)		FAM-5'-caaaagcatcattg + Ct + Gtc + Att-BHQ1
FW	EAV ^b	CATCTCTTGCTTTGTCCTCTAG
as.RV		AGCCGCACCTTCACATTG
probe		5'-Cy5-CGCGCTCGCTGTCAGAACACATTATTGCCACAGCGCG-BHQ3-3'

^a The published sequences of the N gene (GenBank accession number) from RSV A and RSV B which were used for the design of primers and probes are M11486, U39661, U39662, DQ780563 – DQ780569, AY151194 – AY151199, U63644, U50362, AF035006, AY911262, AJ492155 – AJ492167, D00736, NC.001781, AY151200 – AY1512007, AJ492156 – AJ492157, AJ492160 – AJ492161, AF013254, AF013255.

^b Nucleotide sequences of the internal control were obtained from Scheltinga et al. (2005).

plasmids) for inter- and intra-assay variability were estimated using a linear random effects model. For the comparison of independent groups, the Fisher exact test was used for categorical data and the Mann–Whitney U test for continuous data. The Mann–Whitney U test was used to compare the viral load (copies/mL) between the following groups: severe vs. non-severe RSV disease, RSV subgroups A vs. B, single RSV infection vs. co-infection with other respiratory viruses. Severe RSV disease was defined by the need for pediatric ICU admission. All statistical tests were conducted at the two-tailed 5% significance level. Analyses were performed with R 2.9.1 and the contributed R package lme4 (R Foundation for Statistical Computing, Vienna, Austria) and Intercolled Stata 9.2 (College Station, TX, USA).

3. Results

3.1. Assay performance

The analytical limit of detection was determined using six 10-fold serial dilutions of control plasmid in sextuplicate. 100% detection was observed at concentrations ranging from 9.0×10^2 to 9.0×10^6 copies/mL for RSV A and 6.0×10^3 to 6.0×10^7 copies/mL for RSV B. The detection rate was 33% (2/6) for 9.0×10^1 copies/mL of RSV A and 50% (3/6) for 6.0×10^2 copies/mL of RSVB. Significant negative linear relationships were observed between copies/mL and Ct values for the first five 10-fold dilutions from 9.0×10^2 to 9.0×10^6 copies/mL for RSV A (adjusted $R^2 = 0.97$) and 6.0×10^3 to 6.0×10^7 copies/mL for RSV B (adjusted $R^2 = 0.99$). To assess the analytical intra- and inter-assay reproducibility of the amplification step of the assay, Ct values were compared between replicates of five 10-fold serial dilutions tested in the same batch (Table 2) or on different days (Table 3). Coefficient of variation (CVs) for analytical intra-assay variability of RSV A and B ranged from 0.9 to 3.3 and 0.6 to 3.2, respectively. Similarly, CVs for analytical inter-assay variability of both RSV A and B ranged from 1 to 3. Specificity of the assay was confirmed by failure to amplify from 81 specimens known to contain other respiratory viruses (as detected by Seeplex assay). These included influenza viruses ($n = 38$), rhinovirus ($n = 10$), human coronavirus ($n = 14$), human metapneumovirus ($n = 11$), parainfluenza virus ($n = 14$), and adenovirus ($n = 6$). In addition, no amplification was observed for culture supernatants containing high titer influenza viruses.

3.2. Seeplex™ RV detection kit vs. RSV LNA real-time RT-PCR

Among 264 samples, the assay detected significantly more RSV positives than the multiplex Seeplex kit (84/264 [31.8%] vs 64/264 [24.2%], p -value McNemar test < 0.001). The additional 20 samples positive by the RSV LNA assay were confirmed using an RSV G gene RT-PCR (Parveen et al., 2006). None of the samples negative by RSV LNA assay were positive by Seeplex. Fluorescence signals of EAV

internal control were detected for all tested samples at Ct values ranging from 30 to 35.

The viral load of RSV detected in 84 samples ranged from 4.0×10^3 to 10^9 copies/mL (median value 2.9×10^6 ; interquartile range (IQR) 2.7×10^5 to 4.2×10^7). Median (IQR) of viral load in 64 positive samples by both the RSV LNA assay and the Seeplex kit was 8.0×10^6 copies/mL (2.0×10^5 to 5.0×10^7). In the 20 samples positive by RSV LNA assay alone, median (IQR) of viral load was 3.5×10^4 copies/mL (1.7×10^4 to 4.3×10^5). There was a significant difference observed in viral load between RSV positive samples diagnosed by both assays and RSV positive samples by only RSV LNA assay (Mann–Whitney U test, $p = 0.001$) (Fig. 1).

3.3. RSV subgroups, and relationships between viral load and disease severity

Among 84 RSV positive samples, 53 were identified as RSV A (63%) and 31 as RSV B (37%). There was 100% concordance in subgroup identification between the RSV LNA assay and the Seeplex kit. No association was found between RSV subgroups and disease severity, based on the distribution of cases between pediatric intensive care unit (ICU) and other wards. Similarly, no significant differences were observed in viral load between RSV subgroups. Overall, a total of 29% (17/61) of patients with severe presentations (pediatric ICU admissions) were RSV positive, whereas 33% (67/203) of patients on other wards were RSV positive (Fisher's exact test $p = 0.45$). Viral loads in nasal pharyngeal aspirates of pediatric ICU patients were significantly higher when compared to other cases: median (IQR) of viral load (copies/mL) in the 17 pediatric ICU RSV positive samples was 3.8×10^7 (1.6×10^6 to 1.6×10^8) versus 2.5×10^6 (4.5×10^4 to 1.6×10^7) in the 58 positive samples from other wards (Mann–Whitney U test, $p = 0.02$). An association

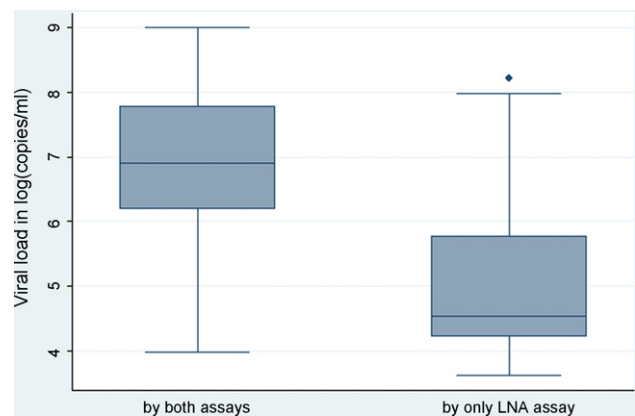


Fig. 1. Viral load detected in RSV positive samples diagnosed by both assays and RSV positive samples by only LNA assay.

Table 2
Analytical intra-assay reproducibility of RSV LNA assay.

RSV A				RSV B			
Plasmid concentration (copies/mL)	Mean Ct values	SD	CV (%)	Plasmid concentration (copies/mL)	Mean Ct values	SD	CV (%)
9.0×10^6	22.4	0.3	1.4	6.0×10^7	24.5	0.5	2.2
9.0×10^5	25.4	0.2	0.9	6.0×10^6	28.3	0.9	3.2
9.0×10^4	29.1	0.3	1.0	6.0×10^5	31.9	0.5	1.6
9.0×10^3	32.7	0.4	1.2	6.0×10^4	35.7	0.5	1.3
9.0×10^2	36.7	1.2	3.3	6.0×10^3	39.3	0.2	0.6

All values are calculated from Ct values of six replicates at each concentration.

SD: standard deviation.

CV: coefficient of variation.

between higher age and lower log-VL was also found (correlation -0.19 ; $p = 0.09$) among RSV positive patients, and younger age was associated with more severe disease (correlation 0.27 , $p = 0.02$). When the dataset was adjusted for age, differences between viral load and severity lost statistical significance (data not shown).

No significant differences in RSV viral load were observed between patients with single infections versus co-infections, or between patients requiring oxygen versus patients who did not.

3.4. Viral co-infection

Detection by Seeplex assay revealed an overall co-infection level of 20% (17/84). These included Influenza viruses ($n = 8$), parainfluenza viruses ($n = 3$), coronaviruses ($n = 1$), adenovirus in ($n = 2$), human metapneumovirus ($n = 3$) and rhinovirus ($n = 1$). The RSV LNA assay revealed an additional 20 RSV positive samples, none of which were coinfecting. The prevalence of coinfection of RSV positive samples was 19% for RSV A (10/53); 23% for RSV B (7/31).

4. Discussion

RSV was the pathogen detected most frequently among hospitalized children with acute respiratory infection in a prospective study conducted between 2004 and 2008 in pediatric ICUs and

respiratory wards of the Hospital for Tropical Diseases in Ho Chi Minh City, Viet Nam (Do et al., 2011). Rapid and sensitive detection of RSV is important for installing infection control measures and thus preventing nosocomial spread, which has been recognized as a major risk in pediatric wards (Macartney et al., 2000). Several RT-PCR based methods and real-time RT-PCR have been described for RSV detection (Borg et al., 2003; Dewhurst-Maridor et al., 2004; do Nascimento et al., 2008; Falsey et al., 2003; Gueudin et al., 2003; Hu et al., 2003; Kuypers et al., 2006, 2004; Mentel et al., 2003; Perkins et al., 2005; van de Pol et al., 2006, 2010; van Elden et al., 2003). An improved PCR diagnostic tool using LNA probes targeting the most conserved region of the N gene was developed, to detect and distinguish simultaneously subgroup A and B and to quantify viral RNA load. An internal control virus (EAV) was added to each specimen in order to monitor efficiency of RNA extraction, reverse transcription, and amplification. This assay exhibited an increased diagnostic yield, from 24% to 32%, compared to a commercial conventional multiplex PCR assay, the Seeplex™ RV detection kit, and produced a diagnostic result within 4 h of sample receipt.

Previous studies have reported higher sensitivities of PCR methods targeting the N gene, compared to methods targeting other genes (Perkins et al., 2005; Rohwedder et al., 1998). Primers and probes were originally designed based on available N gene sequences from NCBI GenBank, including the two reference

Table 3
Precision of RSV real-time PCR: inter-assay reproducibility.

RSV A standard concentration (copies/mL)	Mean Ct-value ^a	Within-run		Between-runs	
		SD ^b	CV ^c (%)	SD ^b	CV ^c (%)
9.0×10^6	22.4	0.2	1	0.0	0
9.0×10^5	25.9	0.2	1	0.7	3
9.0×10^4	29.6	0.3	1	0.4	1
9.0×10^3	33.1	0.3	1	0.4	1
9.0×10^2	36.8	1.0	3	0.0	0
RSV B standard concentration (copies/mL)	Mean Ct-value ^a	Within-run		Between-runs	
		SD ^b	CV ^c (%)	SD ^b	CV ^c (%)
6.0×10^7	24.76	0.2	1	0.27	1
6.0×10^6	25.22	0.6	2	0.22	1
6.0×10^5	31.76	0.5	2	0.20	1
6.0×10^4	35.39	0.6	2	0.64	2
6.0×10^3	38.79	1.0	3	0.80	2

SD standard deviation.

CV coefficient of variation.

Each concentration (from high to low) was run in duplicate (for first two dilutions), quadruplicate (for third dilution) and in sextuplicate (for fourth and fifth dilutions), in each run.

^a Mean was calculated from Ct values of replicates of each concentrations from all days.

^b Standard deviations calculated from Ct values of replicates of each concentrations from all days using a linear random effects model with separate variance components for within- and between-run variation.

^c Coefficient of variation was defined as the SD divided by the mean (%).

strains A2 and B. Twenty-four more recent submissions of N gene sequences from Cambodia showed perfect matches to these primers and probes. This indicates that these primers and probes theoretically could detect all currently known circulating strains of RSV. The 100% detection rate for 9.0×10^2 copies/mL for RSV A and for 6.0×10^3 copies/mL for RSV B are in the same range as previously published methods that used dilution series of plasmid DNA and, when re-calculated and assuming a 1:1 RT efficiency, as methods that used RNA transcripts (ranging from 10^3 to 10^6 copies/mL) (Borg et al., 2003; Gueudin et al., 2003; Kuypers et al., 2004). Significant linear correlations of Ct values and copies/mL were observed at levels of viral load typically found in clinical specimens. In addition, the assay exhibited good analytical reproducibility as evidenced by intra- and inter-assay CVs ranging from 1% to 3%. CVs from other reported diagnostic approaches (based on copies/mL) ranged from 1% to 35.8% (Borg et al., 2003; Gueudin et al., 2003; Kuypers et al., 2004).

As RSV pathogenesis is multifactorial and varied (Collins and Graham, 2007), the relationships between disease severity, viral load and RSV subgroups were studied. Associations between RSV viral load and disease severity have been reported previously (Buckingham et al., 2000; Fodha et al., 2007; Houben et al., 2010), and several studies have definitively shown a negative correlation between RSV viral load and age (Borg et al., 2003; Gueudin et al., 2003; Kuypers et al., 2004). The results of the current study confirm a negative correlation between RSV viral load and age, and significant difference in viral load between severe and non-severe patients (defined by admission status to the pediatric ICU versus the Respiratory ward). However, when the dataset was adjusted for age, differences between viral load and severity lost statistical significance.

Previous studies have also suggested possible differences in pathogenicity between RSV A and B (Campanini et al., 2007; Gerna et al., 2008; Kuypers et al., 2004; Perkins et al., 2005). Here we report no significant differences in viral load or severity between RSV subgroups. Variations in sample collection may also affect observed viral loads, however sample collection for this cohort was conducted by experienced and well-trained hospital personnel using standard operating procedures

In conclusion, this novel real-time RSV LNA assay offers a rapid, specific, highly reproducible and more sensitive alternative (32%) to a commercial multiplex RT-PCR (24%) for RSV detection, and in addition provides quantitation and subgrouping. This information is useful for epidemiological surveillance and clinical decision-making.

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