Multiple-Centre Clinical Evaluation of Rapid Recombinase-Aided Amplification Assays for Five Pathogens

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Research Article

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Abstract

Background: Recombinase-aided amplification(RAA) is a new, simple, and ultrafast isothermal molecular diagnostic technique performed within 30min at 39°C–42°C.In this study, we evaluated the clinical performance of four duplex RAA kits for hepatitis B virus(HBV), human adenovirus 3(HAdV3), human adenovirus 7(HAdV7), and *Bordetella pertussis* and one duplex reverse-transcription RAA (RT-RAA) kit for respiratory syncytial virus (RSV).

Methods: A total of 392 sera and 374 respiratory tract samples were collected from five institutions in four China regions. Each RAA kit's sensitivity and specificity were compared with those of real-time quantitative polymerase chain reaction(qPCR),real-time quantitative reverse-transcription polymerase chain reaction(qRT-PCR), or sequencing.

Results: Compared with qPCR or qRT-PCR, the sensitivities of HBV RAA,RSV RT-RAA, and *B.pertussis* RAA were 97.55%,96.67%, and 100%,respectively,and all of the specificities were 100%.The total coincidence rates were 97.78% (383/392,95%CI:95.63%–98.85%),97.70%(212/217, 95%CI:94.57%–99.16%), and 100%(60/60,95%CI:92.80%–100%),respectively.The Kappa values were 0.977,0.947, and 1,respectively(P<0.05).Regarding the sequencing, the sensitivities of HAdV3 RAA and HAdV7 RAA were 100% and 97.37%, respectively,and all specificities were 100%.The total coincidence rates were 100%(97/97,95%CI:91.58%–100%) and 98.97%(96/97,95%CI:94.39%–99.82%),and the Kappa values were 1 and 0.978 (P<0.05),respectively.

Conclusions: With comparable clinical performance, these RAA kits are suitable assays for rapidly detecting pathogens in resource-limited laboratories.

Background

Infection with pathogens remains a wide spread problem globally and places a severe disease burden on society and individuals[1–3].For example,the human hepatitis B virus(HBV) is a blood-borne pathogen that can cause serious complications, such as liver cirrhosis, liver cancer, and other chronic liver diseases[2].Respiratory tract infection is a common condition caused by various pathogens in people of all ages,especially in infants and young children. Such infection can involve respiratory syncytial virus(RSV), human adenovirus(HAdV), and *Bordetella pertussis*,which is difficult to distinguish only by the clinical symptoms[3–6].Therefore,rapid etiological identification in the early stage is essential for treating, preventing, and controlling the disease.The pathogen isolation and culture and immunological detection methods have limitations in the rapid diagnosis of pathogens in the early stage of the disease because of being time-consuming or having a low detection rate[7–8].Real-time quantitative polymerase chain reaction (qPCR) with high sensitivity and specificity increases clinical laboratories' diagnostic accuracy[7–11].Though commercial PCR-based detection kits and thermal cycling equipment are widely used and improve work efficiency in centralized laboratories,they are challenging to use in resource-limited laboratories because of the need for costly instruments and highly skilled professionals[12–15].The simple and rapid isothermal nucleic acid amplification technology overcomes the difficulty of applying complex PCR technology and instruments and thus is more suitable for grassroots units[16].

Recombinase-aided amplification(RAA),a novel isothermal nucleic acid amplification technique,was reported to detect various pathogens within 30min at 39–42°C in vitro[17–20].Under the activation of magnesium acetate,the primers and recombinase complex searches for and complements the homologous sequence of double-stranded DNA with the help of a single-strand binding protein. The extension of double-stranded DNA is completed under the action of DNA polymerase.With the introduction of reverse transcriptase and a 46–52bp probe, RAA can simultaneously perform reverse transcription and real-time fluorescence detection in a single closed tube[19,21].

In previous studies,we reported rapid duplex real-time RAA detection assays for HBV[22],HAdV3[23], HAdV7[23], and *B. pertussis*[24], and duplex reverse-transcription RAA(RT-RAA)assay for RSV[25].These assays show high sensitivity and specificity and incorporate a non-competitive internal control into the system to prevent false-negative results and increase clinical sample detection accuracy. However,a comprehensive evaluation of the clinical performance of these methods has not yet been carried out.We freeze-dried the primers,probes, and enzymes in reaction unit tubes to make ready-to-use kits.These kits have passed internal quality assessment at the Department of Facility,National Institute for Viral Disease Prevention and Control,the Chinese Center for Disease Control and Prevention (CCDC).In this paper,we report the evaluation of these five RAA kits using a large number of clinical samples from five institutions in four regions of China: Hunan Center for Disease Control and Prevention (CDC) and Hunan Provincial People's Hospital,Beijing Capital Institute of Pediatrics,Suizhou CDC in Hubei,and Tangshan Gongren Hospital in Hebei.

Materials And Methods

Samples

From January 2019 to January 2020,we collected clinical samples from patients in five institutions in four regions of China(Hubei, Hebei, Hunan, and Beijing) for multicenter clinical evaluation.As shown in Table 1,these samples were divided into four groups.Group A: Atotal of 392 serum samples were collected,of which 223 were from Suizhou CDC in Hubei Provinceand 169 were from Tangshan Gongren Hospital in Hebei Province.Group B: A total of 217 sputum and bronchoalveolar lavage fluid samples were collected,of which 121 samples were from Hunan Province CDC and 96 samples were from Capital Institute of Pediatrics in Beijing.Group C: A total of 97 sputum and bronchoalveolar lavage fluid samples were collected from Hunan Province CDC and Hunan People's Hospital.Group D: A total of 60 nasopharyngeal swab samples were collected from the Capital Institute of Pediatrics in Beijing.All aspects of the study were conducted as per the national code of ethics and approved by the institutional review committees of the above-mentioned medical institutions and hospitals.

Table 1
The sample information and the reference methods used in each group

Group		A	B	C	D
Samples type		serum samples	sputum and bronchoalveolar lavage fluid	sputum and bronchoalveolar lavage fluid	nasopharyngeal swab
Sample source(number)		Hebei(169),Hubei(223).	Hunan(121), Beijing(96).	Hunan(97)	Beijing(60)
Reference method		HBV quantitative PCR kit (DAAN GENE, Guangzhou, China)	Respiratory Syncytial Virus (RSV) Real Time rt-PCR Kit(Shanghai ZJ Bio-Tech Co.Ltd.)	Nest PCR and sequencing[26]	B.pertussis DNA detection Kit(Jiangsu Mole Bioscience Co.Ltd)
Result judgment	positive	CT ≤ 40	CT ≤ 38	NA	CT ≤ 36
	negative	CT□ 40	CT□ 38	NA	CT□ 36
Lowest detection limit		10 IU/ml	10^3copies/ml	NA	NA

Nucleic acid extraction

Following the manufacturer's instructions, the total DNA/RNA of all samples was extracted with Tianlong Automatic Extraction Kit (Suzhou Tianlong,China). The nucleic acid was eluted in 80 μL of nuclease-free water and stored at−80°C until use.

Detection of clinical samples using RAA and RT-RAA kits

The serum samples in group A were detected using 2μL of extracted DNA/RNA with the HBV RAA kit, in accordance with a previous report but with a slight modification to the DNA extraction method[22].Samples in group B were detected using 5μL of extracted DNA/RNA with the RSV RT-RAA kit in accordance with a previous report[25].Samples from group C were detected using 2μL of extracted DNA/RNA with the HAdV3 and HAdV7 RAA kits in accordance with a previous assay[23].Nasopharyngeal swabs in group D were detected using 2μL of extracted DNA/RNA with the *B. pertussis* RAA kit in accordance with a previous report[24].Positive controls(recombinant plasmids) of the above five pathogens and negative controls(DNase-free water) were included in each run to ensure the reliability of the experimental results.The primers and probe sequences of the above five RAA kits are shown in Table 2.The FAM channel was used to detect the amplification of the target gene,and the HEX channel was used to detect the amplification of the internal control gene.If both channels were positive or the FAM channel was positive and the HEX channel was negative,the results were considered to be positive.If the FAM channel was negative but the HEX channel was positive,the result was negative.If both channels were negative, the result was considered invalid and the RAA assay was redone.If the samples had discordant results,these samples were retested with the corresponding RAA kit by optimizing the reaction conditions,such as increasing or reducing the amount of input template, or increasing the premixing time of the RAA reaction to fully mix the RAA reaction system.

Table 2
List of primers and probes used in the study

RAA kits	Sequence 5'-3'of Primers and Probes	Reference
HHB RAA	Forward primer 1:ATTCGCAGTCCCCAACCTCCAATCACTCACC Forward primer 2:CTCAATTTTCTAGGGGGAACTACCGTGTGTC Reverse primer:AGCAGGATGAAGAGGAAGATGATAAAACGCC Probe[a]:TCGCAGTCCCCAACCTCCAATCACTCACCAACC[FAMdT]C[THF][BHQ-dT]GTCCTCCAACTTGTC[C3-spacer] Internal control Probe[a]:GTAAGGTGCTAGACTAAAATTGTTGGGACTT [HEXdT]G[THF]A[BHQdT]CTCTGAAGTAAAAGG[C3- spacer]	[22]
RSV RAA	Forward primer 1:TCCYAATTGTATAGCATTCATAGGTGAAGGAGC Reverse primer: TTGCATCTGTAGCAGGAATGGTYAAATTYTCAC Probe[a]:CATCCTGATATAAGATATATTTACAGAAG[FAMdT)][THF][BHQdT]GAAAGATTGCAATGA[C3-spacer] Internal control Probe[a]:GTAAGGTGCTAGACTAAAATTGTTGGGAC [HEXdT][THF][BHQdT]GAATCTCTGAAGTAAAAGG[C3-spacer]	[25]
HADV3 RAA	Forward primer:ATTCCGGCACAGCTTACAATTCACTCGCTCC Reverse primer:TCAGTAGTGG TAATGTCTTT CCCAATTTGC Probe[a]:ACAATGCAGTAACTACCACCACAAACACA[HEXdT][THF][BHQ-dT]GGCATTGCTTCCAT[C3-spacer] Internal control Probe[a]:GTAAGGTGCTAGACTAAAATTGTTGGGACTT [FAMdT]G[THF]A[BHQdT]CTCTGAAGTAAAAGG[C3-spacer]	[23]
HADV7 RAA	Forward primer:ACAACGGGAGAAGACAATGCCACCACATACAC Reverse primer:TCCATCAATATCAGTCCATGATTCTTCTCC Probe[a]:AAGACATTACTGCAGACAACAAGCCCATT[HEXdT][THF][BHQ-dT]GCCGATAAAACATAT[C3-spacer] Internal control Probe[a]:GTAAGGTGCTAGACTAAAATTGTTGGGACTT [FAMdT]G[THF]A[BHQdT]CTCTGAAGTAAAAGG[C3-spacer]	

NOTE:[a]Probe modifications:FAM,6-carboxyfuorescein;HEX,5-hexachlorofuorescein;

THF, tetrahydrofuran; BHQ,black hole quencher;C3-Spacer,30phosphate blocker

RAA kits	Sequence 5'-3'of Primers and Probes	Reference
B.pertussis RAA	Forward primer:AAATCGCCAACCCCCCAGTTCACTCAAGGA Reverse primer:GCACACAAACTTGATGGGCGATCAATTGCT Probe[a]:TGAACACCCATAAGCATGCCCGATTGACCT[FAMdT][THF]C[BHQdT]ACGTCGACTCGAAA[C3-spacer] Internal control Probe[a]:GTAAGGTGCTAGACTAAAATTGTTGGGACTT [HEXdT]G[THF]A[BHQdT]CTCTGAAGTAAAAGG[C3-spacer]	[24]
NOTE:[a]Probe modifications:FAM,6-carboxyfuorescein;HEX,5-hexachlorofuorescein;		
THF, tetrahydrofuran; BHQ,black hole quencher;C3-Spacer,30phosphate blocker		

All of the experimental procedures and biosafety protection measures strictly abided by the regulations on Biosafety Management of Pathogenic Microbiology Laboratories issued by the General Office of the National Health Commission and the working regulations of Clinical Gene Amplification Laboratories[34,35].

Detection of clinical samples using reference methods

Samples in group A,group B, and group D were detected by a commercial HBV quantitative PCR kit(DAAN GENE, Guangzhou, China),Respiratory Syncytial Virus (RSV) Real-Time Reverse-Transcription qPCR Kit(Shanghai ZJ Bio-Tech Co.Ltd.,China), and *B. pertussis* DNA Detection Kit(Jiangsu Mole Bioscience Co.Ltd.,China), in accordance with the manufacturers' instructions.In group C,the nested PCR products of all samples were sequenced and genotyped as previously reported[36]. Information on the above three commercial kits and sequencing is shown in Table 1. PCR was performed in parallel with the use ofthe RAA kits at the facility sites where the clinical samples were provided.All of the above PCR procedures were performed on an ABI 7500 Real-Time PCR Instrument(Applied Biosystems,Foster City,CA)provided by local medical institutions and hospitals.

Results

Sample Characteristics

In group A,392 serum samples were quantified with the commercial HBV qPCR kit, of which 368 samples were HBV-positive with a virus load range from 1.40×10^2IU/ml to 4.00×10^8 IU/ml[cycle threshold(CT) value from 12.82 to 37.80]; the remaining 24 samples were negative.In group B,158 out of 227 samples were positive by the commercial RSV qRT-PCR kit,and the range of CT values was 21.92–38.22.In group C,among 97 HAdV-positive samples by nested PCR and sequencing, there were 53 HAdV3,38 HAdV7,2 HAdV55,2 HAdV2,1 HAdV1,and 1 HAdV4.In group D,50 samples were positive by the commercial *B.pertussis* qPCR kit,and the Ct values ranged from 26.44 to 35.39. The remaining 10 samples were negative.

Evaluation of duplex RAA and RT-RAA kits using clinical samples and comparison with qPCR or qRT-PCR and sequencing

All of the following data are shown in Tables3–6.Among the 392 serum samples in group A,the initial HBVRAA results of 376 samples were consistent with those of qPCR(352 positive and 24 negative)and 16 were inconsistent.The 16 samples with discordant results were rechecked by RAA.By increasing the premixing time to 8 min, five samples from Hubei became RAA-positive.In addition, after the amount of input template was changed from 2 μl to 5 μl,the test results of two samples from Hubei became positive.The RAA retesting results of the remaining nine false-negative samples(1 from Hebei and 8 from Hubei) were consistent with the primary test results.Compared with qPCR,the sensitivity and

specificity of HBV RAA were 97.55% and 100%,respectively.The positive predictive value(PPV) was 100%(95%CI:98.68–100%),the negative predictive value(NPV) was 72.73% (95%CI:54.21–86.06%),the total coincidence rate was 97.78% (383/392)(95%CI:95.63–98.85%),and the Kappa value was 0.977($P < 0.05$).

In group B,the initial RSV RT-RAA results of 207 samples were consistent with those of qRT-PCR(141positive and 66 negative)and 10 were discordant, of which 9 were false negative and 1 was false positive.After rechecking the 10 samples with inconsistent results,one weakly positive RSV RT-RAA sample from Beijing became negative.By extending the premixing time, fourof the nine false-negative samples became true positive.However,the RT-RAA results of five samples remained consistent with the results of the primary test.Compared with the commercial qRT-PCR kit,the sensitivity and specificity of the RSV RT-RAA kit were 96.67% and 100%, respectively.The PPV was 100%(95%CI: 96.78–100%),the NPV was 93.06%(95%CI: 83.86–97.42%),the total coincidence rate was 97.70%(212/217)(95%CI: 94.57–99.16%),and the Kappa value was 0.947($P < 0.05$).

A total of 53 positive samples were detected by the HAdV3 RAA kit and 37 positive samples were detected by the HAdV7 RAA kit in group C; only 1sample of HAdV7 was judged to be false negative.This sample was rechecked and the finding was consistent with the first result.The RAA kits of HAdV3 and ADV7 were negative for other HAdV-positive samples(HAdV1, 2, 4, 55) with a specificity of100%.The sensitivities of HAdV3 and HAdV7 RAA kits were 100% and 97.37%, and the PPVs were 100%(95%CI:91.58–100%) and 100% (95%CI:88.28–100%),respectively.The NPVs were100% (95%CI:90–100%) and 98.33% (95%CI:89.86–99.91%),the total coincidence rates were 100%(97/97) (95%CI:95.43–100%) and 98.97%(96/97,95%CI:94.39–99.82%),and the Kappa values were 1 and 0.978,respectively($P < 0.05$).

Among the 60 samples in group D,50 positive samples and 10 negative samples were detected by the *B.pertussis* RAA kit,which were consistent with the qPCR results. The sensitivity and specificity were both 100%.The PPV was 98.63% (95%CI:91.11–100%),the NPV was 100%(95%CI:65.55–100%),and the total coincidence rate was 100%(95%CI:92.80–100%). The Kappa value was 1($P < 0.05$).

Table 3
Comparison of clinical evaluation of RAA and q-PCR assays to detect HBV

		HBV Duplex RAA Kit		
		positive	negative	total
HBV q-PCR Kit	positive	359	9	365
	negative	0	24	24
	total	359	33	392
Performance evaluation of clinical samples	sensitivity (%)	97.55		
	specificity(%)	100		
	Kappa($p < 0.05$)	0.977		
	The coincidence rate(%)	97.7		
	PPV(%)	100		
	NPV(%	93.06		

Table 4
Comparison of clinical evaluation of RT-RAA and q-PCR assays to detect RSV

		RSV Duplex rtRAA Kit		
		positive	negative	total
RSV q-PCR Kit	positive	145	5	150
	negative	0	67	67
	total	145	71	217
Performance evaluation of clinical samples	sensitivity (%)	96.67		
	specificity(%)	100		
	Kappa($p < 0.05$)	0.947		
	The coincidence rate(%)	97.7		
	PPV(%)	100		
	NPV(%	93.06		

Table 5
Comparison of clinical evaluation of RAA and nested PCR andsequencing assays to detect HAdV3 and HAdV7

		HAdV3 Duplex RAA Kit			HAdV7 Duplex RAA Kit		
		positive	negative	total	positive	negative	total
HAdVnested PCR and Sequencing	positive	50	0	50	50	0	50
	negative	0	10	10	0	10	10
	total	50	10	60	50	10	60
Performance evaluation of clinical samples	sensitivity (%)	100			100		
	specificity(%)	100			100		
	Kappa($p<0.05$)	1			1		
	The coincidence rate(%)	100			100		
	PPV(%)	100			100		
	NPV(%)	100			100		

Table 6
Comparison of clinical evaluation of RAA and q-PCR assays to detect *B. pertussis*

		***B. pertussis* Duplex RAA Kit**		
		positive	negative	total
B. pertussis q-PCR Kit	positive	50	0	50
	negative	0	10	10
	total	50	10	60
Performance evaluation of clinical samples	sensitivity (%)	100		
	specificity(%)	100		
	Kappa($p < 0.05$)	1		

Discussion

Field-applicable pathogen nucleic acid detection is the preferred method of enhancing molecular diagnostic capacity and promotes the early prevention and early treatment of diseases.With the advantages of simple operation, low cost, and minimal device requirements,many isothermal nucleic acid detection techniques[16],including loop-mediated isothermal amplification(LAMP)[26],recombinase polymerase amplification(RPA)[27], and RAA[17,22,25],have demonstrated great potential to be used in low-income countries and regions with limited resources and difficult conditions.LAMP has been successfully applied to the nucleic acid detection of the above five pathogens, with good sensitivity and specificity[26,28–30].However,it is easy to produce false positive results due to cross-reactions associated with the four to six primers used in the method[31].RPA has also been successfully applied to the detection of RSV and HBV,whose sensitivity and specificity are comparable to those of our RAA methods[27,32–33].However, few RPA methods introduce an internal reference to monitor the reaction system, preventing clarification of the authenticity of the nucleic acid detection.

In our work,the introduction of non-competitive internal controls in these five kits greatly reduced the false-negative rate caused by experimental operation errors and system errors[19,25].Two fluorescent probes complementary to the target genes and internal reference quality controls were added to monitor the whole reaction in real time, and the results can be observed within 15–30min[17,22–25].It turned out that the HEX channel had a steady positive curve in all of the experiments in our study, thus confirming the reliability of our results.

We retested and verified the samples whose RAA results were inconsistent with qPCR or qRT-PCR and sequencing results by optimizing the reaction system.We extended the premixing time to 8 min. As a result, five false-negative samples became positive in group A and four false-negative samples became positive in group B.The premixing step is necessary and critical to fully oscillate and mix the RAA reaction system and maximize the likelihood of inter-molecular contact before fluorescence signal detection[19].Therefore, our work confirmed that increasing the premixing time is beneficial to improve the repeatability and rate of nucleic acid detection.In addition,we increased the amount of input template in the HBV RAA system,and two HBV false-negative samples (2.73×10^2IU/ml and 3.45×10^2 IU/ml,respectively)became positive,indicating that more templates might improve the detection rate of the samples with low viral load.In group A,we found that nine negative samples missed by RAA had CT values in the range of 25.29–37.80.Considering that an inhibitor might be present in these samples,we diluted these nine samples and repeated duplex RAA experiments and single RAA experiments(without an internal quality control),yet there were no positive results.We suspect that this might have been due to gene mutation in the RAA primer or probe region, although the exact reasons need to be explored in further work.In the case of group B, five samples were still missed by RAA after retesting, of which three had Ct values

greater than 38,one had a Ct value of 33,and another one 35,suggesting that the RSV RT-RAA kit exhibits slightly low clinical sensitivity.Further improvement should be performed to optimize the RAA system and working conditions.In group C,HAdV3 and HAdV7 kits maintained very high specificity as no cross-reaction was observed with the other four types of HAdV samples(HAdV1, 2, 4, and 55).No false positive or false-negative results were found with the RAA HAdV3 and HAdV7 kits, except for one false-negative sample being misdiagnosed by RAA HAdV7.

We conducted clinical verification in clinical samples from multiple regions in China to test the reliability of the five RAA kits.Among them, the RAA kit for HBV and the RT-RAA kit for RSV were evaluated using clinical samples from two different regions(one in a southern city and the other in a northern city) in China.RAA kits for HAdV3 and HAdV7 were evaluated using clinical samples from two different institutions.No significant difference in the testing results was observed among the different sites of sample collection,indicating wide applicability in testing.Moreover,four different sample types were included in the evaluation(serum, sputum,bronchoalveolar lavage fluid,and nasopharyngeal swab),suggesting that the nucleic acid extraction method in this study works well with these sample types.

Local coworkers carried out all of the tests with the portable RAA fluorescence detector and vortex mixer in the specimen preservation sites.The total turn-around time(from sample in to results out) is 45 min for 16 samples per run per person,while the detection time of the PCR kits used in this experiment using the same samples is more than 70 min.All of the RAA reactions were performed in closed tubes, reducing the likelihood of laboratory contamination.In addition,RAA kits preserve the reaction reagents(enzymes, probes, and primers)in the form of freeze-dried powder,which is easy to transport at room temperature and helpsto reduce the follow-up operation steps[22,24]. The costs of RAA kits are lower than those of the LAMP and PCR assays[21,24].These advantages of RAA kits promote their application in primary laboratories.However,the step of conventional nucleic acid extraction is not skipped in this study, which hinders the field use of RAA kits.Hence, we attempted to simplify the nucleic acid extraction by using a DNA releasing agent atroom temperature and demonstrated that RAA kits for HAdV 3 and 7,HBV,and *B.pertussis* using DNA releasing agent achieved similar results to those obtained using the conventional nucleic acid extraction method(data not shown), which implies the potential for field use of these kits.Another alternative is to integrate nucleic acid extraction with the RAA reaction in a new portable device, which is under development.

Conclusions

With clinical performance comparable to that of commercial qPCR or qRT-PCR assays,the RAA kits in this study are suitable tools for the rapid detection of HBV,RSV,HAdV3,HAdV7, and *B. pertussis* in resource-limited laboratories and also have potential for field use.

Abbreviations

RAA Recombinase-aided amplification

RT-RAA Reverse transcription recombinase-aided amplification

qPCR Quantitative Polymerase Chain Reaction

qRT-PCR Quantitative Reverse transcription Polymerase Chain Reaction

HBV Hepatitis B virus

RSV Respiratory syncytial virus

ADV3 Adenovirus type 3

ADV7 Adenovirus type and 7

B.pertussis Bordetella pertussis

CDC Center for Disease Control and Prevention

PPV Positive predictive value

NPV Negative predictive value

HADV Human adenovirus

LAMP Loop-mediated isothermal amplification

Declarations

Notes

X.S, D.N and H.Z contributed equally to the article.

Ethical approval

All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control,Hunan Center for Disease Control and Prevention and Hunan Provincial People's Hospital,Beijing Capital Institute of Pediatrics,Suizhou CDC in Hubei and Tangshan Gongren Hospital in Hebei.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Conflict of interests

All the authors approved the final manuscript and they have no conflict of interest to declare.

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Contribution of authors

Xuejun Ma, Linqing Zhao, Hao Li designed the study; Xin-xin Shen,Dan-wen Nie,Hong Zhang, Zhi-fei Zhan, Yuan Gao, Rui-huan Wang, Xing-yu Xiang, Xiu-ge Rong, Xin-ying Liu, Xue-ding Bai,Li-li Zhong, Ru-nan Zhu, Fei Xiao, Rui-qing Zhang, Jin-rong Wang, Xin-na Li, Zi-wei Chen, Hao Li,Lin-qing Zhao, Xue-jun Ma performed the experiments; Xinxin Shen, Yuan Gao,

Ruihuan Wang, Ruiqing Zhang, Xueding Bai, Xinna Li, Xuejun Ma analyzed and interpreted the data; Xuejun Ma, Xinxin Shen, Yuan Gao wrote the paper. All authors have read and agreed to the published version of the manuscript.

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