Diagnostic Microbiology and Infectious Disease xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Clinical validation of 3 commercial real-time reverse transcriptase polymerase chain reaction assays for the detection of Middle East respiratory syndrome coronavirus from upper respiratory tract specimens

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ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form 2 January 2017
Accepted 4 January 2017
Available online xxxx

Keywords:
MERS-CoV
UpE
Orf1a
RT-PCR
Saudi Arabia

ABSTRACT

Since discovery of Middle East respiratory syndrome coronavirus (MERS-CoV), a novel betacoronavirus first isolated and characterized in 2012, MERS-CoV real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays represent one of the most rapidly expanding commercial tests. However, in the absence of extensive evaluations of these assays on positive clinical material of different sources, evaluating their diagnostic effectiveness remains challenging. We describe the diagnostic performance evaluation of 3 common commercial MERS-CoV rRT-PCR assays on a large panel (n = 234) of upper respiratory tract specimens collected during an outbreak episode in Saudi Arabia. Assays were compared to the RealStar® MERS-CoV RT-PCR (Alton Diagnostics, Hamburg, Germany) assay as the gold standard. Results showed i) the TIB MolBiol® LightMix *UpE* and *Orf1a* assays (TIB MolBiol, Berlin, Germany) to be the most sensitive, followed by ii) the Anyplex™ Seegene MERS-CoV assay (Seegene, Seoul, Korea), and finally iii) the PrimerDesign™ Genesig® HCoV_2012 assay (PrimerDesign, England, United Kingdom). We also evaluate a modified protocol for the PrimerDesign™ Genesig® HCoV_2012 assay.

1. Introduction

Middle East respiratory syndrome corona virus (MERS-CoV), a novel human betacoronavirus, was first isolated in 2012 following the fatality of a patient with severe acute respiratory infection in Saudi Arabia (Zaki et al., 2012). The genomic sequencing and public release of that isolate (van Boheemen et al., 2012) enabled the development of several real-time reverse transcriptase polymerase chain reaction (rRT-PCR)–based assays to facilitate laboratory detection and confirmation of MERS-CoV RNA in clinical specimens. Screening assays have primarily targeted genomic regions upstream of the envelope gene (*UpE*), while confirmatory assays have targeted viral open reading frames 1a (*ORF1a*), *ORF1b*, or both (*ORF1ab*) (Corman et al., 2012a, b). More recently, alternative genomic targets have included the nucleocapsid (N) (Lu et al., 2014) and RNA-dependent RNA polymerase genes (Corman et al., 2012b). Following deployment of these molecular assays, reports of MERS-CoV cases continue to rise both regionally and globally. According to WHO statistics, as of 31 August 2016, a total of 1800 laboratory confirmed MERS-CoV cases including 640 related deaths have been reported in 27 countries. Rapid and reliable detection of MERS-CoV is therefore essential to controlling the spread of this emerging threat.

In the event of a MERS-CoV outbreak, the World Health Organization (WHO) have authorized emergency use of 2 rRT-PCR assays for MERS-CoV detection, i) the RealStar® MERS-CoV RT-PCR assay (Corman et al., 2014), a commercially available assay produced by Altona Diagnostics (Hamburg, Germany) and ii) the CDC Novel Coronavirus 2012 Real-time RT-PCR assay (Lu et al., 2014), distributed free of charge to qualified laboratories by the Centers of Disease Control and Prevention (Atlanta, GA, USA). During the initial stages of MERS-CoV spread, these assays enabled rapid diagnosis to support the public health response to MERS-CoV. However, as with other currently available commercial assays, evaluation of the clinical sensitivities of these kits has been limited to virus spiked mock samples or small numbers of clinical specimens from MERS-CoV infected cases. Due to the recent increase in commercially available MERS-CoV rRT-PCR kits (n = 16) (Pas et al., 2015), there is a need to evaluate assay performances on different specimen types to better understand their diagnostic effectiveness.

Lower respiratory tract (LRT) specimens are known to contain high viral loads. However, upper respiratory tract (URT) specimens such as

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http://dx.doi.org/10.1016/j.diagmicrobio.2017.01.003
0732-8893/

nasopharyngeal/oropharyngeal (NPOP) swabs remain the easiest and most common specimen type collected from patients experiencing respiratory distress. This article describes the performance evaluation of 3 commonly used commercial MERS-CoV rRT-PCR assays and 1 modified protocol, on a large panel of URT specimens collected during the period surrounding an outbreak episode in Riyadh, Saudi Arabia.

2. Materials and methods

2.1. Specimens

During the late winter to early spring period of 2015, King Khalid University Hospital (KKUH), Riyadh (Saudi Arabia) observed an outbreak of MERS-CoV infections. Specimens were screened by RT-PCR using the RealStar® MERS-CoV rRT-PCR assay. Laboratory-confirmed samples were also subjected to deep sequencing and phylogenetic analysis as previously described (Somily et al.).

For this study, archived combined NPOP swabs in viral transport medium (VTM; Copan, Brescia, Italy) from patients presenting with probable or suspected diagnosis of MERS-CoV, attending KKUH in the period surrounding the outbreak (1 January and 30 April 2015) were obtained from the Molecular Diagnostic Unit, KKUH. This included 34 archived NPOP swabs (in VTM) from 18 sequence-confirmed MERS-CoV–positive cases, and a further 200 randomly selected MERS-CoV–negative NPOP swabs. Aliquots of VTM from the original frozen NPOP tubes of these specimens were prepared in 2.0-mL tubes. All specimens were delinked from patient details, reissued unique study identifiers, reextracted, and concurrently subjected to MERS-CoV rRT-PCR as described below. The researcher performing testing was blinded to the original MERS-CoV screening outcomes of these 234 specimens. The RealStar® MERS-CoV rRT-PCR assay was used as the gold standard. The results of the RealStar® MERS-CoV assay in Tables 2–3 represent the results from the reextracted specimens, for comparative analysis.

An additional 22 diverse clinical and proficiency testing specimens, previously positive for other respiratory viruses using the Anyplex™II RV16 detection assay (Seegene, Seoul, Korea) (Section 2.4.1), were also included in this study to evaluate assay cross-reactivity. This study was approved by, and performed according to the guidelines of The KKUH and College of Medicine institutional review board committee under project number E16–1886.

2.2. RNA extraction

Total nucleic acid extractions from VTM aliquots were performed on the MagNA Pure Compact system (Roche Applied Science, Bavaria, Germany), using the Nucleic Acid Isolation Kit I and the default instrument settings. Extractions were performed on 300 μL of each specimen, with a final elution volume of 50 μL. Duplicate extractions were performed for each specimen and the final elutions are pooled into one 100-μL extract for concurrent use.

2.3. MERS-CoV rRT-PCR assays

Table 1 summarizes the specifications of all the MERS-CoV rRT-PCR assays used in this study. Amplifications were performed according to the manufacturer's instructions. All assays were supplied as complete kits, inclusive of master mix reagents and internal control, with the exception of the TIB MolBiol® LightMix kits (TIB MolBiol, Berlin, Germany), which can be used with the LightCycler® Multiplex RNA Virus Master Mix (Roche Applied Science) (Assay 2). Assay signatures (except Anyplex™ Seegene, discussed below) targeted the *UpE* genomic region for MERS-CoV screening, and the *Orf1a* or *Orf1ab* genes for confirmatory investigations. Positive *UpE* and *Orf1a* amplification confirmed MERS-CoV infection. For the purposes of this study, all specimens were analyzed on both the screening and confirmatory assays. Additionally, internal controls were spiked in the PCR mixtures according to each manufacturer's instructions.

A modification of the PrimerDesign™ Genesig® assay was also evaluated. The unmodified assay (assay 3A) reaction composition was as follows: 10 μL of Oasig™ One-Step 2× qRT-PCR Master Mix (Primer Design) was added to 1 μL of HCoV_2012 primer/probe mix, 1 μL of internal extraction control primer/probe mix, and 8 μL of the extracted sample RNA. For the controls, only 5-μL control material was used, and the reaction volume adjusted with water. Amplification was performed on the LightCycler® 480 system using the thermocycling conditions specified by GeneSig®. For the modified protocol (assay 3B), the LightCycler® Multiplex RNA Virus Master Mix was used in the place of

Table 1
MERS-CoV rRT-PCR assay specifications.

Characteristic	Assay 1	Assay 2	Assay 3A	Assay 3B (modified 3A)	Assay 4
Manufacturer/ Assay details	RealStar® MERS-CoV RT-PCR assay	TIB MolBiol® LightMix ModularDx Kit Coronavirus SA1 (Erasmus Medical Center [EMC]) upstream E-gene assay and TIB MolBiol® ModularDx Kit MERS-Coronavirus (EMC) *Orf1a* assay	PrimerDesign™ Genesig® Kit for Human Coronavirus 2012 (HCoV_2012) assay (PrimerDesign, England, United Kingdom)	PrimerDesign™ Genesig® Kit for Human Coronavirus 2012 (HCoV_2012) assay	Anyplex™ Seegene MERS-CoV detection assay
Master Mix supplier (if different)		Roche Realtime Ready RNA Virus Master (Roche Applied Science)		Roche Realtime Ready RNA Virus Master	
Assay signature	*UpE* *Orf1a*	*UpE* *Orf1a*	*ORF5/E* *ORF1ab*	*ORF5/E* *ORF1ab*	Not disclosed
Internal control	Yes	No	Yes	Yes	Yes
Nucleic acid input volume[a]	10 μL	5 μL	8 μL	5 μL	5 μL
Total reaction volume[a]	25 μL	20 μL	20 μL	20 μL	25 μL
Limit of detection[b]	*Orf1a*: 0.93 copies/μL *UpE*: 0.54copies/μL	10 copies/reaction	<100 copies/reaction	Not determined	Not disclosed
Thermal cycler	Qiagen Rotor-Gene Q® 3000 (Germantown, MD, USA)	Roche LightCycler® 2.0 Instrument (Roche Applied Science)	Roche LightCycler® 480 (Roche Applied Science)	Roche LightCycler® 480	BioRad CFX (Hercules, CA, USA)
PCR time	2.5 h	45–50 min	2.5 h	2.5 h	2.5 h
Analysis type	Semi-quantitative	Semi-quantitative	Quantitative	Semi-Quantitative	Qualitative

[a] *UpE* and *Orf1a* reactions were performed separately.
[b] Limit of detection values is manufacturer-specified values.

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the Oasig™ Master Mix as follows: 20-μL reaction volumes were set up containing 6.25 μL of 5× RT qPCR reaction mix, 0.5 μL of RT enzyme, 1 μL of the PrimerDesign™ Genesig® HCoV_2012 primer/probe mix, 6.25 μL of nuclease-free water, and 5 μL of extracted sample RNA or control material. Amplification and thermocycling were as described above.

At the time of performing this study, the Anyplex™ Seegene qualitative assay was commercially available but still in the validation stages, and the details of the target were not disclosed. However, during the preparation of this manuscript, Seegene has since released a new CE in vitro diagnostics (IVD) marked semi-quantitative MERS-CoV assay (to replace the first generation assay) based on detection of the popular *UpE* and *Orf1a* targets to enable both screening and confirmation of MERS-CoV infection as described above.

Additionally, TIB MolBiol® have also released a new CE-IVD marked assay which detects *UpE*, *Orf1a*, and now "*N*" gene and incorporates an internal control to be added during the extraction stage to monitor the entire process from nucleic acid extraction to amplification. However, their original assay (described here) is still commercially available and used for MERS-CoV diagnostics.

2.4. Detection of other respiratory viruses

2.4.1. Anyplex™II RV16 detection assay

Assay cross-reactivity was evaluated using 9 proficiency testing specimens obtaining from the College of American Pathologists and a further 13 clinical specimens previously identified as positive for respiratory viruses other than MERS-CoV. The Anyplex™ II RV16 detection assay (Seegene) was used to confirm the presence of 16 respiratory viruses including adenovirus, influenza A and B viruses, parainfluenza viruses 1–4, human rhinovirus, respiratory syncytial viruses A and B, human bocavirus, metapneumovirus, coronavirus 229E, coronavirus NL63, coronavirus OC43, and enterovirus. Briefly, total nucleic acid was extracted as described in Section 2.2 above, except that an extraction (internal) control was added to each specimen. During the reverse transcription stage, complementary DNA (cDNA) was synthesized using the cDNA Synthesis Premix kit (Seegene). Two microliters of random hexamer and 8 μL of extracted RNA were added to prealiquoted tubes containing 10-μL cDNA synthesis master mix and amplified according to the manufacturers' instructions. Once complete, real-time respiratory virus detection reactions were set up using the RV16 TOM-A and TOM-B sets. These 2 master mixes together enable detection of the above 16 respiratory viruses and internal control. Duplicate 20-μL reaction volumes were set up containing 8 μL of cDNA, 5 μL of 4× RV16 Primers (TOM-A or TOM-B), 5 μL of 4× Anyplex™ master mix, and 2-μL water. Amplification and detection were performed on the CFX96™ system (BioRad) under the following conditions: activation at 50 °C for 4 minutes; denaturation at 95 °C for 15 minutes, followed by 50 cycles of 95 °C for 30 seconds; 60 °C for 1 minute, and 72 °C for 30 seconds. Catcher melting temperature analysis (CMTA) was performed by cooling the mixture to 55 °C, maintaining the mixture at 55 °C for 30 seconds, and heating the mixture from 55–85 °C. CMTA was performed 3 times during amplification, after cycle numbers 30, 40, and 50. Fluorescence was continuously measured during the temperature increase stage. Finally, melt-peak analysis was performed by Seegene Viewer software (Seegene).

3. Results

Evaluation of the diagnostic performances of the 3 commercial MERS-CoV rRT-PCR assays showed the TIB MolBiol® LightMix assays as having the best clinical sensitivity, followed by the Seegene and GeneSig® assays (Table 2). Comparative cycle threshold (C_T) values are detailed in Table 3, except for the Anyplex™ Seegene assay which provided only qualitative detection. All internal controls (where applicable) were valid, with minimal C_T variations.

The TIB MolBiol® LightMix assay displayed 94.12% (32/34) sensitivity in our clinical specimen pool. Two of the 34 specimens yielded i) positive *UpE* gene amplification, without amplification of the confirmatory *Orf1a* target and ii) positive *Orf1a* gene amplification, without amplification of the *UpE* target (Table 3), both resulting in a negative MERS-CoV classification. The Anyplex™ Seegene assay displayed a modest sensitivity of 79.4% (27/34). However, the original GeneSig® assay (assay 3A) lacked sensitivity (41.2%; 14/34), particularly with specimens displaying low viral loads (high C_T values on comparative assays). Comparatively, our modification of the GeneSig® protocol (assay 3B) improved MERS-CoV RNA detection to 73.5% (25/34).

Of 200 randomly selected MERS-CoV–negative respiratory specimens from the same collection period, all assays displayed negative rRT-PCR results. Additionally, during the cross-reactivity evaluation using 22 specimens positive for other respiratory viruses, the specificity of both the Anyplex™ Seegene and GeneSig® assays was 100% (Table 4). Only the TIB MolBiol® LightMix assay produced limited cross-reactivity with 1 influenza B–positive clinical specimen (confirmed by the Anyplex™ II RV16 assay).

Under our blind-testing protocol, all extracted RNA samples used in this study were also retested on the RealStar® MERS-CoV rRT-PCR assay (the gold standard), and C_T values are also displayed in Table 3. All 34 known positive specimens yielded positive results, indicating that archiving at −80 °C (4–6 months) had not compromised specimen integrity. The RealStar® assay in most cases (76%) yielded lower C_T values than all other assays. This assay also displayed 0% cross-reactivity with other respiratory viruses.

4. Discussion

Presently, RT-PCR detection of MERS-CoV RNA remains the most reliable method for laboratory confirmation of infection. However, an actual "gold standard" is yet to be classified, with most assays relying on the 2 gene *UpE*/*Orf1a* signatures for screening and confirmation of MERS-CoV RNA. This comparative analysis of MERS-CoV rRT-PCR assays evaluates their clinical sensitivity and presents their C_T values to enable

Table 2
Performance summary of MERS-CoV assays with clinical specimens screened during retrospective surveillance.

Protocol	% Sensitivity[a]	% Specificity[a]	% Positive predictive value (95% CI)	% Negative predictive value (95% CI)
Assay 1 (RS)	100 (34/34)	100 (222/222)	100 (89.72–100)	100 (98.35–100)
Assay 2 (LM)	94.12 (32/34)	99.55 (221/222)	96.97 (84.24–99.92)	99.10 (96.80–99.89)
Assay 3A (GS)	41.18 (14/34)	100 (222/222)	100 (76.84–100)	91.74 (87.52–94.88)
Assay 3B (GS-M)	73.53 (25/34)	100 (222/222)	100 (86.28–100)	96.10 (92.73–98.20)
Assay 4 (ASG)	79.41 (27/34)	100 (222/222)	100 (87.23–100)	96.94 (93.80–98.76)

CI = confidence interval; RS = RealStar; LM = LightMix; GS = GeneSig; GS-M = GeneSig modified protocol; ASG = Anyplex Seegene.
[a] Value in parentheses represents the number of positives/total number of true positives (sensitivity) or the number of negatives/total number of true negatives (specificity).

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Table 3
Comparative performance of MERS-CoV assays on positive clinical specimens.

Sample no.	Assay 1 (C_T)[a] RS		Assay 2 (C_T)[a] LM		Assay 3A (C_T)[a] GS		Assay 3B (C_T)[a] GS-M		Assay 4 ASG
	UpE	*Orf1a*	*UpE*	*Orf1a*	*ORF5/E*	*Orf1ab*	*ORF5/E*	*Orf1ab*	
S1	22.77	22.43	24.61	25.47	33.72	36.26	28.09	34.65	POS
S2	28.12	27.23	30.20	30.18	35.63	38.86	32.22	37.36	POS
S3	23.4	22.69	25.31	25.61	34.3	38.47	28.35	34.62	POS
S4	16.35	14.61	21.86	16.46	25.72	28.47	18.77	25.91	POS
S5	27.72	27.17	(34.59)	NEG	37.58	NEG	NEG	NEG	NEG
S6	36.73	32.39	35.5	35.26	NEG	NEG	33.63	NEG	NEG
S7	29.30	27.47	31.65	31.73	NEG	NEG	30.77	37.15	POS
S8	27.61	27.53	32.24	33.92	NEG	NEG	(36.82)	NEG	POS
S9	28.11	28.3	32.10	31.03	(39.36)	NEG	(36.85)	NEG	POS
S10	29.95	28.05	29.67	28.51	(37.61)	NEG	(33.84)	NEG	POS
S11	27.58	27.15	22.89	23.47	32.05	31.70	30.6	30.76	POS
S12	31.31	29.3	32.71	32.09	NEG	NEG	32.66	33.51	POS
S13	28.17	27.94	25.92	26.93	33.63	39.91	24.07	26.48	POS
S14	30.53	29.87	21	22	29.94	30.59	22.59	24.48	POS
S15	30.39	28.85	29	30	NEG	NEG	(37.13)	NEG	POS
S16	27.33	27.14	30.06	30.42	(35.71)	NEG	31.06	32.68	POS
S17	27.82	28.11	31.95	32.20	39.53	48.75	31.51	33.01	POS
S18	27.51	28.14	29.91	30.80	(40.51)	NEG	31.58	33.13	POS
S19	27.01	27.35	19.97	19.78	27.54	28.20	24.64	27.3	POS
S20	28.13	28.31	35.5	35.5	NEG	NEG	33.11	34.03	NEG
S21	27.57	27.47	30.64	31.05	NEG	NEG	33.97	34.03	NEG
S22	27.52	27.61	31.27	31.34	NEG	NEG	30.81	33.09	POS
S23	27.58	27.85	32.3	32.7	(38.83)	NEG	33.12	34.75	NEG
S24	20	18.02	23.75	24.13	30.01	32.74	23.86	30.13	POS
S25	20.78	18.75	23.28	23.59	29.36	31.7	23.89	31.46	POS
S26	33.36	31.13	34.19	33.12	NEG	NEG	(35.23)	NEG	POS
S27	21.72	22.15	22.67	21.89	30.11	29.20	24.32	27.29	POS
S28	22.77	20.52	25.91	25.66	32.61	35.89	25.07	32.29	POS
S29	36.32	35.34	35.59	32.58	NEG	NEG	NEG	NEG	NEG
S30	28.69	28.32	NEG	(21.41)	NEG	NEG	16.78	34.52	NEG
S31	19.18	17.79	20.53	21.23	28.24	31.59	21.67	29.48	POS
S32	33.11	30.7	34.20	34.87	NEG	NEG	(37.64)	NEG	POS
S33	27.09	25.75	34.5	33.5	(37.61)	NEG	32.63	38.33	POS
S34	30.07	28.90	35.21	33.44	NEG	NEG	31.7	34.64	POS
Internal control (C_T)	26 ± 3 °C		NA		31 ± 2 °C		30 ± 3 °C		Tm = 66–66.5 °C

RS = RealStar; LM = LightMix; GS = GeneSig; SG = Seegene; NA = not applicable; Tm = melting temperature; POS = positive; NEG = negative.
[a] Parentheses have been used to highlight single gene positive results.

Table 4
Assay cross reactivity with respiratory viruses other than MERS-CoV.

Virus*	Source**	Assay 1 RS	Assay 2 LM	Assay 3A GS	Assay 3B GS-M	Assay 4 ASG
MPV	PT specimen	NEG	NEG	NEG	NEG	NEG
RSVA	PT specimen	NEG	NEG	NEG	NEG	NEG
HRV	PT specimen	NEG	NEG	NEG	NEG	NEG
FluA	PT specimen	NEG	NEG	NEG	NEG	NEG
PIV1	PT specimen	NEG	NEG	NEG	NEG	NEG
RSVA	PT specimen	NEG	NEG	NEG	NEG	NEG
Coronavirus 229E	PT specimen	NEG	NEG	NEG	NEG	NEG
Coronavirus OC43	PT specimen	NEG	NEG	NEG	NEG	NEG
Coronavirus NL63	PT specimen	NEG	NEG	NEG	NEG	NEG
FluA	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
FluB	Clinical Specimen	NEG	POS (CT: 36.19/36.13)	NEG	NEG	NEG
PIV3/FluB	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
FluA	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
AdV/HRV	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
PIV4/AdV/HRV	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
RSVA	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
AdV/HRV	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
AdV/RSVA	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
RSVA/HRV/HEV	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
RSVA/AdV	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
HRV	Clinical Specimen	NEG	NEG	NEG	NEG	NEG
AdV/RSVB	Clinical Specimen	NEG	NEG	NEG	NEG	NEG

* MPV, Human Metapneumovirus; HRV, Human Rhinovirus; Flu, Influenza Virus; PIV, Parainfluenza Virus; HEV, Human Enterovirus; AdV, Adenovirus; RSV, Respiratory Syncytial Virus.
** PT, Proficiency Testing material (College of American Pathologists).

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a robust comparison of diagnostic assay performances on NPOP specimens under our testing conditions. Although C_T values from different assays can be affected by various assay components and platforms used, our analyses of 34 URT specimens showed the TIB MolBiol® LightMix assays to be the most sensitive (94.12%), followed by the Anyplex™ Seegene and GeneSig® protocols.

Discordant results between the assays were seen with those specimens containing low virus concentrations (high C_T values), reflecting differences in the limits of detection (LOD). Evaluating the LOD is critical to preventing underdiagnoses of MERS-CoV infections, and should be a consideration when selecting a commercial assay for routine diagnostics. For these purposes, the European Virus Archive has produced quantitative standards for *UpE* and *Orf1a*. However, we were unable to obtain these standards during this investigation. Nevertheless, the TIB MolBiol® LightMix and GeneSig® protocols (Assays 2–3) produced some single gene–positive (indeterminate) results, which in a clinical diagnostic setting would require further confirmation by screening a secondary specimen (ideally from the LRT where a higher viral load is expected) before overall interpretation as "MERS-CoV negative". Additionally, the GeneSig® and Anyplex™ Seegene assays also produced several negative classifications for our sequence-confirmed specimens. No such indeterminate or negative results were observed with the RealStar® assay.

Although the TIB MolBiol® LightMix assay was the most sensitive assay secondary to RealStar®, it displayed limited cross-reactivity with influenza B, which needs to be investigated further. However, it is the Kingdom of Saudi Arabia's policy to perform parallel screening for MERS-CoV and Influenza (A, B, and H1N1) in suspected MERS-CoV cases. For this reason, it would be easy to identify any potential cross-reactivity with this assay. Nevertheless, the TIB MolBiol® assay was the most efficient of all the assays, yielding amplification results within 45–50 minutes. This would be highly advantageous during an outbreak situation where turnaround time is critical.

The reduced sensitivity of the GeneSig® assay is thought to be a result of the suboptimal performance of the lyophilized RT enzyme. As GeneSig® primers detect common MERS-CoV targets (*UpE*, *Orf1ab*), Whitaker et al. proposed that assay performance may improve by replacing the lyophilized 1-step RT-PCR with a nonlyophilized counterpart. We agree with this conclusion as using the LightCycler® Multiplex RNA Virus Master Mix with the GeneSig® primers (Assay 3B) significantly improved assay sensitivity from 41.2–73.5%.

According to a recently published study evaluating the first MERS-CoV external quality assessment (EQA) panel of 16 reported commercial rRT-PCR assays used by participants for routine diagnostics, the GeneSig® and RealStar® MERS-CoV assays included in our study were among the most popular (Pas et al., 2015). The EQA panel of 12 samples including 7 MERS-CoV–positive samples gave the RealStar® MERS-CoV rRT-PCR assay a sensitivity score of 100% and GeneSig® 83–92%. Our findings support this superior sensitivity of the RealStar® MERS-CoV rRT-PCR assay and the reduced sensitivity of the GeneSig® assay, even with our URT specimens. Reanalysis of our known positive specimens on the RealStar® MERS-CoV rRT-PCR assay showed consistently lower C_T values for 76% (26/34) of specimens, enabling reliable MERS-CoV detection when other assays approached their "diagnostic cutoff". However, this could possibly be attributed to the larger nucleic acid input volume and amplification reaction volumes relative to the other assays.

A further study (Kim et al., 2016) evaluated the analytical and clinical sensitivity of 6 commercial MERS-CoV rRT-PCR assays used in Korea. Only the LightMix assay was common with our study, although it was utilized on a different PCR platform (LightCycler 480 versus our LightCycler 2.0). The other 5 assays used in that study highlight the diversity in MERS-CoV test availability between Korea and Saudi Arabia. Kim et al. (2016) were able to utilize an updated version of the Anyplex™ assay (although pre-CE-IVD approval), which detected *UpE* and internal control (versus version 1 of the assay used in our study which incorporated melt-peak analysis only). Nine LRT sputum specimens were used to determine assay sensitivities, a further 9 LRT specimens were used to evaluate the effects of PCR inhibition on positive results, and 28 nasopharyngeal swabs were used to evaluate assay cross-reactivity. The overall sensitivity and specificity of all 6 assays were deemed sufficient for diagnostic use. However, sputum specimens were shown to be particularly PCR inhibition prone, negatively impacting assay sensitivity, and highlighted the importance of optimizing pretreatment and RNA extraction procedures when using this specimen type. In comparison, PCR inhibition was not observed in the NPOP specimens used in the present study, and minimal variations were observed in the internal control C_T values. Nevertheless, the C_T values for the LightMix assay reported from sputum specimens (Kim et al., 2016) were comparable with those of our URT specimens.

A limitation of this study was evaluation of only URT specimens. The WHO recommends the preferential collection of LRT specimens (e.g., broncheoalveolar lavage and tracheal aspirate) for MERS-CoV molecular diagnostics as they contain higher viral loads. However, LRT specimens are traditionally only collected from patients requiring hospitalization. Nevertheless, where this is not possible, screening URT specimens are still acceptable and are of diagnostic value, as further demonstrated by our results.

This report investigates the diagnostic accuracy of 3 commercial MERS-CoV rRT-PCR assays on NPOP swabs. Although ultimately MERS-CoV diagnosis cannot rely on a single assay, studies such as ours may contribute toward better understanding the diagnostic performance of currently available MERS-CoV rRT-PCR assays. The RealStar®, LightMix, and GeneSig® assays described are still widely used in the Kingdom of Saudi Arabia, as is the MagNA pure compact automated nucleic acid extraction system. Our study further supports the *UpE*/*Orf1a* genomic regions as well-chosen amplification targets for MERS-CoV diagnostics. There is, however, a need to develop more rapid diagnostic assays with shorter turnaround times.

Acknowledgments

The authors would like to thank the following KKUH Molecular Diagnostic Unit staff for their contribution to this work: Mr. Nasr Sinjilawi, Mrs. Fatimah Alanazi, Mr. Mukhtar Almutowa, and Mr. Muhammad-Ismail Hamayati. This project was supported by the College of Medicine Research Chair, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia. The authors declare no conflicts of interests.

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Please cite this article as: Mohamed DH, et al, Clinical validation of 3 commercial real-time reverse transcriptase polymerase chain reaction assays for the detection of Middle E..., Diagn Microbiol Infect Dis (2017), http://dx.doi.org/10.1016/j.diagmicrobio.2017.01.003