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External quality assessment for the molecular detection of MERS-CoV in China

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ABSTRACT

Background: In May 2015, an imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection occurred in China, so rapid and reliable diagnosis of suspected cases was necessary.
Objectives: An external quality assessment (EQA) program for the molecular detection of MERS-CoV was organized by the National Center for Clinical Laboratories (NCCL).
Study design: MS2 virus-like particles (VLPs) encapsulating specific RNA sequences of MERS-CoV were prepared as positive specimens. The assessment panel, which comprised of three negative and seven positive samples with different concentrations of VLPs, was distributed to 56 laboratories from 16 provinces, municipalities, or autonomous regions for molecular detection.
Results: Among the received data sets, three employed an in-house-developed real-time reverse-transcription polymerase chain reaction (rRT-PCR) assay and the others applied various commercial rRT-PCR kits. Overall, the majority of laboratories (46/56, 82.1%) could achieve 100% accuracy for MERS-CoV detection, but three laboratories (5.4%) still had room for improvement. Consequently, all negative samples were identified correctly, reaching 100% specificity. The false-negative rate was 3.1%, and most of the false-negative results were obtained from samples with relatively low concentration, indicating an urgent need to improve detection in weak-positive specimens.
Conclusions: The majority of participants possessed reliable diagnostic capacity for the detection of MERS-CoV. Moreover, EQA is indispensable because it can help enhance the diagnostic capability for the surveillance of MERS-CoV infections and allow comparison of the results among different laboratories.

1. Background

Since the discovery of the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 in Saudi Arabia [1,2], there have been 1621 laboratory- confirmed human cases, including 584 deaths, with a 36.0% mortality rate [3]. MERS-CoV is a kind of novel enveloped virus with a single-stranded, positive-sense RNA genome, which can cause severe respiratory complications and renal failure in infected patients [2,4]. Although most MERS-CoV infections occurred in the Middle East, cases have been reported in Europe, North America, and Asia through people traveling from the Middle East or their contacts [5]. Since May 2015, the Republic of Korea has newly been affected, causing the largest outbreak of MERS-CoV outside the Arabian Peninsula [6]; this highlighted the importance of a surveillance system to prevent further spread [7]. Simultaneously, China increased its efforts to detect MERS-CoV because of an imported case of MERS from Korea, which was a threat to public health [8]. Hence, rapid and effective diagnosis of the infectious agent is crucial for the timely control and management of this disease.

Laboratory detection of MERS-CoV is based on molecular techniques, and the most commonly used approach is real-time reverse-transcription polymerase chain reaction (rRT-PCR), which is an essential and indispensable method for laboratory molecular diagnostics [9–11]. Recently, the World Health Organization (WHO) published an updated interim guidance for laboratory testing of MERS-CoV [12]. Three assays for diagnosing MERS-CoV

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infections have been developed, which were based on the detection of specific regions of viral RNA by rRT-PCR. The tests include an assay targeting upstream of the envelope gene (upE) [10], which is highly sensitive and recommended for laboratory screening, followed by confirmation with assays targeting either the open reading frame 1b (ORF 1b) [10] or 1a (ORF 1a) [11]. Meanwhile, the United States Centers for Disease Control and Prevention (US CDC) developed rRT-PCR assays targeting the nucleocapsid (N) gene of MERS-CoV to complement the upE and ORF 1a/1b assays during screening and confirmation [6,13]. Additionally, confirmation for the screening test can be aided by partial sequencing of the RNA-dependent RNA polymerase (RdRp) or N gene [11].

Since the first case of MERS was identified, the Chinese CDC has published and provided primer-probe sets for the rapid molecular diagnosis of suspected infections, as well as several commercial kit manufacturers. However, the diversity of nucleic acid extraction techniques, detection methods, instruments and commercial assays used, and disparate individual operators may cause variations in evaluating laboratory performance. Therefore, there is an urgent need for quality assurance with diagnostics, which could make results obtained from different laboratories comparable. External quality assessment (EQA) is an important part of laboratory quality management and improvement.

2. Objectives

A nationwide EQA was implemented by the National Center for Clinical Laboratories (NCCL) to evaluate the MERS-CoV diagnostic capability of laboratories within the Chinese mainland.

3. Study design

3.1. Sample preparation

Two target segments of nucleotide sequences from the MERS-CoV genome (GenBank accession no. JX869059.2) were synthesized separately: the first was 394 bp, consisting of part of the upE gene, and the other was 1629 bp, which encompassed part of the ORF 1a, ORF 1b, N, and RdRp genes. The chimeric sequences were amplified (primers listed in Table 1), gel-purified, and separately subcloned into *Kpn* I/*Pvu* I and *Kpn* I/*Pac* I sites of the pACYC-MS2 vector [14,15] to form the recombinant plasmids, pACYC-MS2-upE and pACYC-MS2-ORF 1ab/N, respectively. These two kinds of MS2 virus-like particles (VLPs) packaging specific sequences of MERS-CoV were expressed and purified according to previously published protocols of our laboratory [14–16]. Then, they were identified by transmission electron microscopy (TEM), enzymatic digestion test, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and RT-PCR.

3.2. Evaluation of MS2 VLPs

The stability of the two types of MERS-CoV VLPs was evaluated in Dulbecco's modified eagle medium (DMEM, Gibco; USA) under various conditions. Firstly, RNA of the VLPs was extracted using the QIAamp viral RNA Mini kit (Qiagen, Germany) and quantified using an absorbance-based nucleic acid quantification method (NanoDrop 2000; Thermo). The concentration of sample was calculated using the formula: $(6.02 \times 10^{23}$ copies/mol) × (g/mL)/(MW g/mol) = copies/mL. Each kind of quantified VLPs was diluted with DMEM in order to obtain samples with two concentrations. The samples were then incubated at 4 °C, 25 °C, and 37 °C for a defined period until each time point and stored at −80 °C until all of the samples were collected. The samples were assessed in duplicate using the MERS-CoV rRT-PCR kit (BioPerfectus Technologies, Jiangsu, China), with DMEM being used as a negative control in each run.

3.3. EQA organization

The coded panel ($n = 10$) for EQA consisted of three negative and seven positive samples, ranging from 2.4×10^4 to 4.8×10^7 copies/mL, in accordance with the detectable RNA concentrations in the clinical respiratory specimens [17]. The positive samples were obtained by appropriate dilution and mixing of the two kinds of MERS-CoV VLPs (Table 2). Among the seven positive samples, No. 1503 had the lowest concentration, which was prepared to determine the detection limit of various commercial kits and detection capability of different laboratories. Two replicate specimens (No. 1506 and No. 1508) were prepared to evaluate the repeatability of each participant's operation. DMEM was used for negative samples and as dilution buffer for positive ones. In addition, one negative sample (No. 1502) containing measles virus (MV) and hepatitis C virus (HCV) VLPs was used to test whether MERS-CoV detection could be obscured by the presence of other viruses. Before dispatch, each sample was assessed and identified three times by ourselves.

Test samples were shipped on ice to participating laboratories, along with the instructions. All participants were asked to detect MERS-CoV by using their routine operating procedures. Details, such as qualitative results and the threshold cycle (Ct) value of each sample, information about RNA extraction and detection methods, instruments used, and the time point of beginning MERS-CoV molecular detection, were recorded.

3.4. Statistical analysis

The results were classified as competent (100% correct responses), acceptable (≤2 incorrect results), or improvable (>2 incorrect results). All statistical analyses were performed using SPSS version 19.0. Sensitivities among different groups were compared by applying Pearson's chi-square test or Fisher's exact test when appropriate with P values <0.05 regarded as statistically significant.

4. Results

4.1. Evaluation of the sample panel

MERS-CoV VLPs were constructed and expressed successfully, which were validated through sequencing and a series of experiments (Fig. S1). As shown in Fig. S1A, the diameters of the MERS-CoV VLPs were about 28–30 nm, which was confirmed by TEM. A 14-kDa protein band could be seen when conducting the SDS-PAGE analysis (Fig. S1B). After digestion with RNase A and DNase I for 1 h at 37 °C, a single band (about 1 kb in length) was visible using 1% agarose gel electrophoresis with ethidium bromide (Fig. S1C). RT-PCR was applied to confirm the successful packaging of the target sequences (Fig. S1D). Stability evaluations revealed that the MERS-CoV VLPs could be stable for at least two weeks at 37 °C, one month at 25 °C, and two months at 4 °C (Fig. S2).

Each sample of the panel was assessed by using different commercial kits before distribution, and all the tests could confirm positive and negative results successfully.

4.2. Participants and methodologies

In total, 56 laboratories from 16 provinces, municipalities, or autonomous regions participated in the EQA for MERS-CoV molecular detection. Among them, 34 laboratories were from the provincial or municipal CDC of China, 13 laboratories were from