EXTERNAL QUALITY ASSESSMENT OF MOLECULAR DIAGNOSTICS FOR ZIKA VIRUS AND MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS IN KOREA

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Abstract. In Korea since August 2016, molecular tests for Zika virus and Middle East respiratory syndrome coronavirus (MERS-CoV) have been extended for use in non-governmental medical institutions to prepare for potential emergence of these viruses. The study evaluated reliability of Zika virus and MERS-CoV molecular tests performed by non-governmental medical laboratories. In December 2016, information on participation in a Zika virus and MERS-CoV external quality assessment (EQA) was sent to 36 laboratories performing molecular tests for these viruses. Sensitivity and specificity were tested using five samples of Zika virus and 12 samples of MERS-CoV, each containing a different viral load. Thirty-three laboratories returned their EQA results for Zika virus and 25 for MERS-CoV. For Zika virus assay, 73% of participating laboratories tested both serum and urine samples, and for MERS-CoV, 92% tested lower, or both upper and lower, respiratory tract specimens. There was one false-positive result from Zika virus tests. MERS-CoV EQA results were 96% correct except for one outlier. EQA samples for Zika virus and MERS-CoV tests showed good homogeneity and stability for up to 72 hours at 4°C and eight weeks at -70°C. Although there is still room for improvement with respect to outliers and false-positive results, EQA of Zika virus and MERS-CoV molecular tests in Korea revealed good assay performance. The EQA method used in the study will help laboratories cope with assays of other new pathogens.

Keywords: external quality assessment, Middle East respiratory syndrome coronavirus, quality control, Zika virus, Korea

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INTRODUCTION

Babies born to mothers infected with Zika virus can present with microcephaly (Mlakar *et al*, 2016) and/or neurologic disorders such as Guillain-Barré syndrome (Dos Santos *et al*, 2016); indeed, this was observed in a global outbreak of 2016 (Musso and Lanteri, 2017). In Korea, since the first case in March 2016, 28 cases have been confirmed (KCDC, 2018). In

2015, there was a large outbreak of Middle East respiratory syndrome coronavirus (MERS-CoV) infection in South Korea (Kim *et al*, 2017), with 185 laboratory-confirmed cases and 36 deaths (WHO, 2015).

In order to control future outbreaks of Zika virus and MERS-CoV infections, prompt detection of these viruses is essential. In August 2016, the Korean Centers for Disease Control and Prevention (KCDC) approved performing Zika virus and MERS-CoV molecular tests by non-governmental laboratories, leading to a rapid expansion of molecular testings for Zika virus and MERS-CoV within a short period of time (KCDC, 2016). Non-governmental laboratories have carried out molecular tests using diagnostic kits, which were rapidly approved. Thus, an external quality assessment (EQA) was needed to evaluate quality and reliability of the assays in current use so that appropriate corrective actions can be undertaken for under-performing laboratories.

This study reports results on the production of reliable and stable external quality control materials for use in EQA of molecular tests of Zika virus and MERS-CoV, as well as on appraisal of proficiency of Zika virus and MERS-CoV molecular tests performed by non-governmental medical laboratories.

MATERIALS AND METHODS

Participating laboratories

In December 2016, information regarding participation in a Zika virus and MERS-CoV EQA was sent to 36 laboratories performing semi-quantitative RT-PCR tests for detection of Zika virus and MERS-CoV.

As the study was designed as a survey and EQA without any human data, institutional review board approval was not required.

EQA materials

Materials used to test for Zika virus and MERS-CoV were produced by KCDC. In brief, BHK-21 cells (ATCC, Manassas, VA) were inoculated with Zika virus MR-766 strain (ATCC) and cultured at 37°C under a humidified atmosphere containing 5% CO_2 for 3 days. Viral infection was confirmed by assessing cytopathic effects and RT-PCR using a PrimerDesign Zika Real-Time RT-PCR standard kit (PrimerDesign, Southampton, UK). Virus titer was 6.7×10^5 copies/5 μl of RNA with an RT-PCR cycle threshold (Ct) of 19. Virus was inactivated for 5 minutes at 60°C (Muller *et al*, 2016). EQA panel for Zika virus comprised five samples: a positive control (Z1), three serially diluted samples (Z2, Z3, and Z4) and a negative control (Z5). Z1 was generated from a 5-fold dilution of Zika virus material in standard refeeding medium containing 2% fetal bovine serum (Quidel, San Diego, CA); Z2, Z3, and Z4 were 5-fold serially diluted in matrix (pooled urine), and Z5 was pooled urine sample lacking Zika virus. EQA samples were shipped on dry ice according to KCDC regulations for transport of highly dangerous pathogens and infectious substances (KCDC, 2015).

For MERS-CoV, Vero cells (ATCC CCL-81, American Type Culture Collection) were inoculated with MERS-CoV KNIH002 strain (KCDC) and cultured at 37°C under 5% CO_2 atmosphere for 5 days. Infection was confirmed by assessing cytopathic effects and by RT-PCR using a PowerChek MERS Real-Time PCR kit (Kogene Biotech, Seoul, Korea). Titer of MERS-CoV was 2.2×10^5 pfu/ml and a Ct value of 20–21. Virus was inactivated at 65°C for 30 minutes (Leclercq *et al*, 2014). Dilution matrix for upper respiratory tract samples was pooled

nasopharyngeal aspiration sample and for lower respiratory tract samples pooled bronchoalveolar lavage sample. All pooled samples were negative for respiratory viruses. EQA panel comprised 12 samples: a positive control (MB1 for the lower respiratory panel and MN1 for the upper respiratory panel), three samples of three different virus concentrations for the upper respiratory panel (MN2, MN3 and MN4) and three for the lower respiratory panel (MB2, MB3, and MB4), a negative control (MB5 and MN5 for lower and upper respiratory panel, respectively), and *in vitro*-transcribed *upE* (M6) and *ORF1a* (M7) RNAs produced using a T7 promoter system (Tabor and Richardson, 1985; Seong *et al*, 2016). MB1 and MN1 were generated by diluting inactivated MERS-CoV 5-fold in standard refeeding medium containing 2% fetal bovine serum (Quidel). MN5 and MB5 was nasopharyngeal aspiration and bronchoalveolar lavage sample, respectively, lacking MERS-CoV. Virus samples contained three different virus concentrations, namely, high (MN2, MB2), medium (MN3, MB3), and low (MN4, MB4), prepared by 5-fold serial dilution of inactivated MERS-CoV in pooled nasopharyngeal aspiration or bronchoalveolar lavage sample. EQA samples were shipped as described above.

In order to validate stability and homogeneity, EQA panel samples were assayed using a RealStar Zika Virus RT-PCR kit (Altona Diagnostics, Hamburg, Germany) and a PowerChek MERS Real-time PCR kit (Kogene Biotech). For assessing homogeneity, six panels were picked at random and assayed three times; and for stability, samples were maintained at 4°C for 24, 48 and 72 hours, and at -70°C for 14 days and for 2 months, then three panels from each storage conditions were picked at random and assayed three times.

Statistical analysis

Ct values were compared among laboratories and tests. Median value, values of first quartiles and third quartiles, mean Ct value, and standard deviation (SD) were used to analyze EQA results. For homogeneity and stability tests, median value and percent coefficient of variation (% CV) for each sample were analyzed and assumption of homogeneity of variance was tested using Levene's test of equality of variances employing Microsoft Excel 2016 (Microsoft, Redmond, WA). Satisfaction of homogeneity and stability was accepted if % CV <5% and p-value ≥ 0.05.

RESULTS

Participation of laboratories

For EQA survey, 34/36 (94%) laboratories returned their EQA results, with 33/34 (97%) laboratories for Zika virus, 25 (73%) for MERS-CoV, 24 (70%) for both Zika virus and MERS-CoV EQA, nine (26%) for Zika virus only, and one (3%) for MERS-CoV only.

RT-PCR methods and turn-around time

For Zika virus detection, 24/33 (73%) laboratories employed only RealStar Zika Virus RT-PCR kit (Altona Diagnostics), 8 (24%) only AccuPower ZIKV Real-time RT-PCR kit (Bioneer, Daejoen, Korea) and one (3%) both kits, each kit having its own controls. Of the 25 RealStar Zika Virus RT-PCR kit (Altona Diagnostics) users, 12 (48%) extracted DNA using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), five (20%) using MagnaPure kit (Roche Diagnostics, Mannheim, Germany), three (12.0%) used easyMAG (BioMérieux, Marcy l'Étoile, France), two (8%) using TANBead kit (Taiwan Advanced Nanotech, Taoyuan City, Taiwan), and one each (4%) using Easy-Spin Total RNA kit (iNtRON,

Sungnam, Korea), QiaAmp DSP Virus kit (Qiagen) and SeePrep kit (Seegene, Seoul, Korea). Of the nine users of AccuPower ZIKV Real-time RT-PCR kit (Bioneer), six (67%) employed ExiPrep Dx Viral DNA/RNA kit (Bioneer), and one each (11%) MagnaPure kit (Roche Diagnostics), QiaAmp DSP Virus kit (Qiagen) and TANBead kit (Taiwan Advanced Nanotech). Twenty-four (73%) laboratories assayed both serum and urine samples, eight (24%) only urine samples and one (3%) only serum samples. Turn-around time for Zika virus was ≤8 hours for three (9%) laboratories, >8 - ≤24 hours for 16 (47%), >24 - ≤48 hours for seven (21%) laboratories, and >48 hours for seven laboratories.

For MERS-CoV testing, 22 (88%) laboratories used only PowerChek MERS Real-time PCR kit (Kogene Biotech), two (8%) laboratories only AccuPower MERS-CoV Real-Time RT-PCR kit (Bioneer), and one (4%) laboratory both kits. The former has its own controls and the latter uses human GAPDH gene as spiked control. For nucleic acid extraction, of the 23 PowerChek MERS Real-time PCR kit (Kogene Biotech) users, seven (30%) employed QIAamp Viral RNA Mini kit (Qiagen), six (26%) MagnaPure kit (Roche Diagnostics), three (13%) easyMAG kit (BioMérieux), three TANBead kit (Taiwan Advanced Nanotech), and one each (4%) chemagic Viral NA/gDNA Kit (Perkin Elmer Chemagen, Baesweiler, Germany), Geno-Prep FFPE DNA Kit (Genolution Pharmaceuticals, Seoul, Korea), ExiPrep Dx Viral DNA/RNA kit (Bioneer), and SeePrep (Seegene) (4.3%). Two (67%) of the three users of AccuPower MERS-CoV Real-Time RT-PCR kit (Bioneer) employed ExiPrep Dx Viral DNA/RNA kit (Bioneer) and the remaining laboratory QIAamp Viral RNA Mini kit (Qiagen). All laboratories homogenized lower respiratory tract specimens prior to MERS-CoV testing, with 17 (68%) adding phosphate-buttered saline to a physical homogenization method and three (12%) adding proteinase K. Ten (40%) laboratories assayed only lower respiratory specimens and 13 (52%) both lower and upper respiratory specimens. Turn-around time was ≤8 hours for five (20%), >8 - ≤24 hours for 18 (72%) laboratories and >24 - ≤48 hour for two (8%) laboratories.

EQA performance

Ct value (mean ± SD) for Z1-Z4 sample was 17 ± 2, 21 ± 4, 24 ± 3, and 27 ± 3, respectively (Fig 1). Thirty-two (94%) laboratories reported Z5 sample as negative but one laboratory reported a false-positive result.

Ct value (mean ± SD) for M6 was 34 ± 2. Ct value (mean ± SD) for *upE* of MN1 - MN4 sample was 23 ± 3, 24 ± 3, 26 ± 3, and 28 ± 3, respectively with MN5 sample yielding negative results from all 25 laboratories (Fig 2A), Ct value (mean ± SD) for *upE* of MB1 - MB4 sample was 23 ± 3, 24 ± 3, 26 ± 3, and 29 ± 3, respectively, and MB5 sample was reported negative by all laboratories; however, one laboratory reported outlier Ct value for MB3 and MB4 of 35 and 35, respectively.

With respect to M7, Ct value (mean ± SD) was 34 ± 2. Ct value (mean ± SD) for *ORF1a* of MN1 - MN4 samples was 24 ± 3, 24 ± 3, 26 ± 3, and 29 ± 3, respectively, and again, MN5 sample was reported negative by all laboratories (Fig 2B). Ct value (mean ± SD) for *ORF1a* of MB1 - MB4 sample was 24 ± 3, 25 ± 3, 27 ± 3, and 29 ± 3, respectively, with MB5 sample negative from all laboratories.

Homogeneity and stability of EQA samples

Median Ct value for Z1 - Z4 samples assayed using RealStar Zika Virus RT-PCR kit (Altona Diagnostics) was 14, 18, 20,

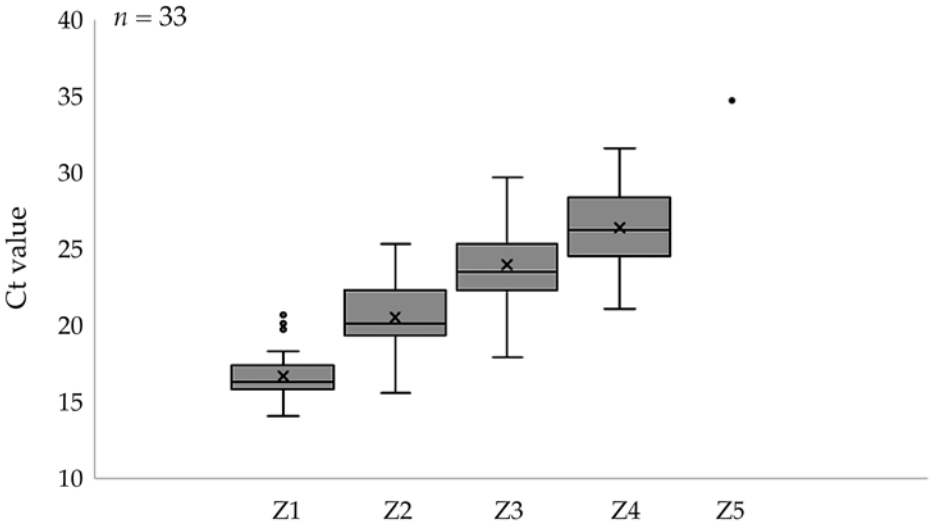

Fig 1-Semi-quantitative RT-PCR cycle threshold (Ct) values for Zika virus samples reported by thirty-three non-governmental laboratories. Z1, positive control (5-fold dilution of Zika virus); Z2-Z4, 5-fold serially diluted Zika virus samples; Z5, negative control (vehicle lacking Zika virus). Box denotes Ct values, horizontal line within each box median value, vertical line range. x = mean. Dot = outlier.

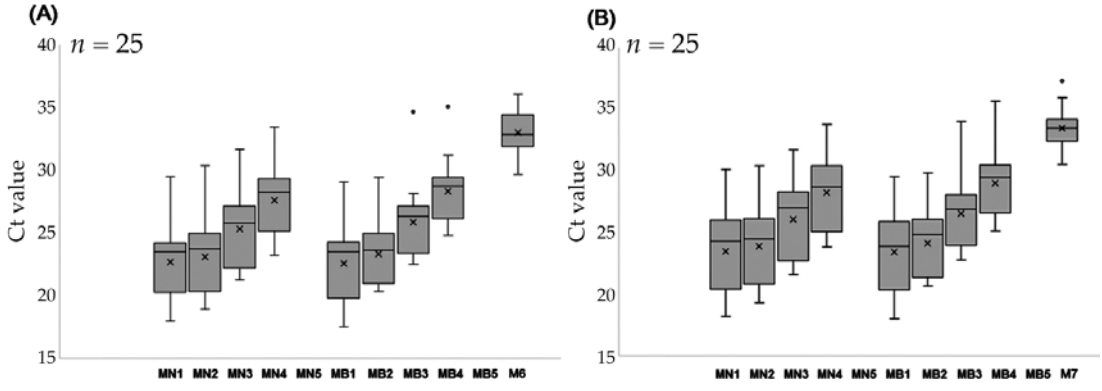

Fig 2-Semi-quantitative RT-PCR cycle threshold (Ct) values for Middle East respiratory syndrome coronavirus (MERS-CoV) *upE* (A) and *ORF1a* (B) reported by twenty-five non-governmental laboratories. MB, lower respiratory assessment panel: MB1, positive control (5-fold dilution of inactivated MERS-CoV); MB2-MB4, 5-fold serial dilution of inactivated MERS-CoV; MB5, negative control (bronchoalveolar lavage lacking MERS-CoV). MN, upper respiratory assessment panel for MERS-CoV: MN1, positive control (5-fold dilution of inactivated MERS-CoV); MN2-MN4, 5-fold serial dilution of inactivated MERS-CoV; MN5, negative control (nasopharyngeal aspiration lacking MERS-CoV). M6, *in vitro*-transcribed *upE* RNA; M7, *in vitro*-transcribed *ORF1a* RNA. Box denotes box and whisker plot of Ct values, horizontal line within each box median value, vertical line range. x = mean. Dot = outlier.

and 23, respectively, with % CV of 1.5, 2.6, 4.9, and 4.1, respectively. All *p*-values were >0.05. Median Ct value for *upE* assayed using PowerChek MERS Real-time PCR kit (Kogene Biotech) was 24, 26, 28, and 32 for MN1 - MN4 samples, respectively, with % CV of 1.7, 1.8, 1.8, and 2.8, respectively. Median Ct value for MB1 - MB4 was 24, 25, 26, and 29, respectively, with % CV of 1.2, 1.7, 1.5, and 2.7, respectively. Median Ct value for *ORF1a* assayed using PowerChek MERS Real-time PCR kit (Kogene Biotech) was 24, 26, 29, and 32 for MN1 - MN4 samples, respectively, with % CV of 1.5, 1.3, 1.3, and 1.9, respectively; while that for MB1 - MB4 samples were 24, 24, 27, and 29, respectively, with % CV of 1.4, 1.4, 1.2, and 2.5, respectively. All *p*-values were >0.05.

Ct values obtained for Zika virus EQA panels after storage at 4°C for 24, 48 and 72 hours or at -70°C for 1, 2, 4, and 8 weeks were within acceptable ranges, with % CVs <5 (data not shown). Ct values obtained for *upE* and *ORF1a* MERS-CoV EQA panel were also within acceptable ranges, with % CVs <5% (data not shown).

DISCUSSION

Many factors play a role in emerging and re-emerging infections (Abubakar *et al*, 2016), and laboratories may encounter new pathogens. Performing diagnostic tests for emerging pathogens solely in governmental laboratories requires time, *eg*, for shipping specimen and processing. This may delay diagnosis, isolation of suspected cases and treatment. Thus, extension of diagnostic testing for emerging pathogens to non-governmental laboratories has many advantages, such as more rapid turn-around time and treatment. Such outsourcing was indeed used as an emergency measure in South Korea for Zika virus and MERS-CoV (KCDC, 2016); however, there was no formal validation of testing for Zika virus and MERS-CoV in these laboratories. All laboratories that replied to the survey conducted agreed allowing molecular testing for Zika virus and MERS-CoV to non-governmental laboratories helped to prevent spread of infectious diseases and extending permission of non-governmental laboratories to such testing was needed when new pathogens emerge.

In order to ensure reliable test results, EQA is essential, particularly when using unapproved diagnostic kits. The first Zika virus EQA was performed for European expert laboratories in 2016 revealing a need for improvement in EQA for molecular detection of Zika virus, with only 40% of laboratories scoring well and the majority of incorrect results being false positives (Charrel *et al*, 2017). In the present EQA study, only one laboratory (out of 33) reported a false positive result for Zika virus. The laboratory in question has since improved environmental decontamination of pre- and post-testing and taken steps to educate medical technologists of test guidelines and sharing of problems occurrence sheet.

For MERS-CoV testing, the first international EQA was conducted in 2014, and all participating laboratories are able to detect MERS-CoV samples, although 8% of the laboratories reported false positive results (Pas *et al*, 2015). South Korea conducted EQA of MERS-CoV testing in 2015 revealing a significant difference in Ct values for the same samples tested using commercial kits (Seong *et al*, 2016). However, during an outbreak in 2015 all 47 participating laboratories in EQA reported 100% correct results for blind test samples (Seong *et al*, 2016). In the present study, one laboratory (out of 25) reported outliers with high Ct values. The

cause was traced to the RNA extraction kit, which was remedied when a different nucleic acid extraction was employed. That only one laboratory reported outlier results is reassuring as the majority of non-govermewntal laboratories would be expected to provide correct diagnosis.

All EQA samples were adequately homogeneous and stable on storage at 4°C and at -70°C. Thus affirming the reliability of the virus panels used in the EQA tests.

In summary, to the best of our knowledge this is the first survey and EQA of Zika virus and MERS-CoV molecular detection carried out in non-govermental laboratories that perform molecular tests. EQA allowed identification of participating laboratories (constituting a very small minority) with technical problems, which were readily identified and subsequently corrected. The study supports performing regular EQA of current and future non-govermental laboratories involved in diagnosing emerging and re-emerging viral diseases in the country, and will serve as a basis for strengthening the capacity of civilian laboratories to deal with new pathogens in Korea and other countries.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Abubakar I, Stagg HR, Charles T, Rodrigues LC. Infectious disease epidemiology. 1st ed. Oxford: Oxford University Press, 2016.

Charrel R, Mogling R, Pas S, *et al*. Variable sensitivity in molecular detection of Zika virus in European expert laboratories: external quality assessment, November 2016. *J Clin Microbiol* 2017; 55: 3219-26.

Dos Santos T, Rodriguez A, Almiron M, *et al*. Zika virus and the Guillain-Barré syndrome - case series from seven countries. *N Engl J Med* 2016;375:1598-601.

Kim KH, Tandi TE, Choi JW, Moon JM, Kim MS. Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak in South Korea, 2015: epidemiology, characteristics and public health implications. *J Hosp Infect* 2017; 95: 207-13.

Korea Centers for Disease Control and Prevention (KCDC) Public Health Weekly Report. Safe transportation management for domestic infectious substances. Seoul: KCDC, 2015. [Cited 2015 Oct 8]. Available from: http://www.cdc.go.kr/CDC/cms/content/mobile/96/65896_view.html

Korea Centers for Disease Control and Prevention (KCDC). Guidelines for the molecular diagnosis of Middle East respiratory syndrome coronavirus and Zika virus for non-governmental laboratories in Korea. Seoul: KCDC, 2016. [Cited 2016 Aug 29]. Available from: http://www.cdc.go.kr/board.es?mid=a20504000000&bid=0014&tag=&act=view&list_no=128370

Korea Centers for Disease Control and Prevention (KCDC) Public Health Weekly Report. An epidemiological survey for Zika virus infection cases in Korea, 2016-2017. Seoul: KCDC, 2018. [Cited 2018 Feb 22]. Available from: https://www.cdc.go.kr/CDC/eng/info/CdcKeWreport.jsp?menuIds=HOME002-MNU0576-MNU0583&q_type=&year=2018&cid=78957&pageNum=1

Leclercq I, Batéjat C, Burguiere AM, Manuguerra JC. Heat inactivation of the Middle

East respiratory syndrome coronavirus. *Influenza Other Respir Viruses* 2014; 8: 585-6.

Mlakar J, Korva M, Tul N, *et al*. Zika virus associated with microcephaly. *N Engl J Med* 2016; 374: 951-58.

Muller JA, Harms M, Schubert A, *et al*. Inactivation and environmental stability of Zika virus. *Emerg Infect Dis* 2016; 22: 1685-7.

Musso D, Lanteri MC. Emergence of Zika virus: where does it come from and where is it going to? *Lancet Infect Dis* 2017; 17: 255.

Pas SD, Patel P, Reusken C, *et al*. First international external quality assessment of molecular diagnostics for Mers-CoV. *J Clin Virol* 2015; 69: 81-5.

Seong MW, Lee SJ, Cho SI, *et al*. External quality assessment of Mers-Cov molecular diagnostics during the 2015 Korean outbreak. *Ann Lab Med* 2016; 36: 230-4.

Tabor S, Richardson CC. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* 1985; 82: 1074-8.

World Health Organization (WHO). Middle East respiratory syndrome coronavirus – surveillance archive. Geneva: WHO, 2015. [Cited 2018 Nov 8]. Available from: http://www.who.int/westernpacific/emergencies/surveillance/archives/mers