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Development and validation of different indirect ELISAs for MERS-CoV serological testing

Anwar M. Hashem[a,b,*], Sawsan S. Al-amri[a], Tagreed L. Al-subhi[a], Loai A. Siddiq[a], Ahmed M. Hassan[a], Maha M. Alawi[a,b,c], Rowa Y. Alhabbab[a,d], Salwa I. Hindawi[a,e,f], Osama B. Mohammed[g], Nabil S. Amor[g], Abdulaziz N. Alagaili[g], Ahmed A. Mirza[d], Esam I. Azhar[a,d]

[a] Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia
[b] Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[c] Infection Control & Environmental Health Unit, Faculty of Medicine, King Abdulaziz University Hospital, Jeddah, Saudi Arabia
[d] Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
[e] Blood Transfusion Services, King Abdulaziz University Hospital, Jeddah, Saudi Arabia
[f] Department of Hematology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[g] KSU Mammals Research Chair, Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

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ABSTRACT

Since 2012, MERS-CoV has caused up to 2220 cases and 790 deaths in 27 countries with Saudi Arabia being the most affected country with ~83.1% of the cases and ~38.8% local death rate. Current serological assays such as microneutralization (MN), plaque reduction neutralization, immunofluorescence, protein microarray or pseudoparticle neutralization assays rely on handling of live MERS-CoV in high containment laboratories or need for expensive and special equipment and reagents and highly trained personnel which represent a technical hurdle for most laboratories in resource-limited MERS-CoV endemic countries. Here, we developed, compared and evaluated three different indirect ELISAs based on MERS-CoV nucleocapsid protein (N), spike (S) ectodomain (amino acids 1–1297) and S1 subunit (amino acids 1–725) and compared them with MN assay. The developed ELISAs were evaluated using large number of confirmed seropositive (79 samples) and seronegative (274 samples) MERS-CoV human serum samples. Both rS1- and rS-ELISAs maintained high sensitivity and specificity (≥90%) across a wider range of OD values compared to rN-ELISA. Moreover, rS1- and rS-based ELISAs showed better agreement and correlation with MN assay in contrast to rN-ELISA. Collectively, our data demonstrate that rS1-ELISA and rS-ELISA are more reliable than rN-ELISA and represent a suitable choice for seroepidemiological testing and surveillance in MERS-CoV endemic regions.

1. Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel zoonotic lineage C Beta-coronavirus that was first identified in 2012 in Saudi Arabia (Zaki et al., 2012). This virus causes disease ranging from asymptomatic infections to mild or severe respiratory disease which is usually followed by multi-organs failure and death in the elderly and individuals with comorbidities. As of April 2018, MERS-CoV has caused up to 2220 laboratory-confirmed cases in 27 countries with 35.6% fatality rate (790 deaths) (WHO, 2018). Most index MERS patients from non-endemic regions have had a travel history to the Middle East (de Groot et al., 2013). So far, Saudi Arabia has reported 1844 cases (~83.1% of total) with 716 deaths (~38.8% local death rate) (WHO, 2018). Current evidence suggests that MERS is a zoonotic infection most likely from dromedary camels (Alagaili et al., 2014; Azhar et al., 2014) which is usually followed by limited human-to-human spread and outbreaks in healthcare or household settings. One large cross-sectional study from Saudi Arabia suggested low seroprevalence of < 0.15% of MERS-CoV in the general population compared to 2.3% and 3.6% in high-risk groups such as shepherds and slaughterhouse workers, respectively (Müller et al., 2015).

Several MERS-CoV serological assays have been developed and

* Corresponding author at: Special Infectious Agents Unit, King Fahd Medical Research Center, Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, P.O. Box 80205, Jeddah 21589, Saudi Arabia.
E-mail address: amhashem@kau.edu.sa (A.M. Hashem).

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utilized in epidemiological and surveillance studies especially in endemic regions. Serological detection of MERS-CoV antibodies (Abs) in patients is based on screening by indirect ELISA using MERS-CoV nucleocapsid (N) protein and confirmation by immunofluorescence assay (IFA) of MERS-CoV infected cells and/or microneutralization (MN) assay (Al-Abdallat et al., 2014). Indirect ELISA based on recombinant MERS-spike (S) ectodomain protein (amino acids 1–1297) was recently included in this algorithm (Trivedi et al., 2017). Other groups have also used several other assays such as S protein microarray, IFA staining based on cells expressing recombinant full length S protein, plaque reduction neutralization test (PRNT), MERS-CoV pseudoparticle neutralization (ppNT) assay, and indirect ELISA based on S1 recombinant protein (Perera et al., 2013; Reusken et al., 2013; Zhao et al., 2013; Hemida et al., 2014; Meyer et al., 2014; Fukuma et al., 2015; Grehan et al., 2015; Müller et al., 2015; Muth et al., 2015; Park et al., 2015; Chan et al., 2017). More recently, S-based competitive ELISA (cELISA) based on neutralizing monoclonal Abs (mAbs) have been developed and shown to correlate well with neutralization assays with high sensitivity and specificity (Fukushi et al., 2017).

Most of the aforementioned serological assays are associated with several drawbacks and limitations especially in endemic areas. For example, gold standard neutralization methods such as MN and PRNT require high containment laboratories which are limited or not available in endemic regions. Furthermore, protein microarray, cELISA, IFA and ppNT require expensive and special equipment and reagents, and/or highly trained technical staff which represent a technical hurdle for most laboratories in the Middle East. On the other hand, indirect ELISA is simple, rapid, cheap, and does not require high containment facilities making it a suitable choice especially in resource-limited regions. However, there is a limited number of reports testing human clinical samples mainly due to the lack of well-characterized human sera. Therefore, the goal of this study was to develop, validate and compare in house developed indirect ELISAs for the detection of MERS-CoV Abs based on recombinant N, S1 and S proteins using a large number of well characterized human serum samples.

2. Materials and methods

2.1. Cell line and virus

African Green monkey kidney-derived Vero E6 cells (ATCC #1568) were grown and maintained as previously described (Al-Amri et al., 2017). Human MERS-CoV isolate (MERS-CoV/Hu/Taif/SA/2015) (Al-Amri et al., 2017) was passaged and titrated by tissue culture infection dose 50 ($TCID_{50}$) assay in Vero E6 cells as previously described (Coleman and Frieman, 2015).

2.2. Samples

A total of 100 archived serum samples obtained from healthy blood donors and confirmed to be negative for MERS-CoV, as tested by live virus MN assay, were used to determine the preliminary cut-off values for the developed indirect ELISAs. The positive control was a serum sample collected from a confirmed MERS patient in a previous study (Azhar et al., 2014). The second set of specimens included 353 serum samples collected between 2014 and 2017 from high-risk groups which included slaughterhouse workers and camel handlers in Makkah, Qassim and Riyadh provinces. These samples included 79 seropositive samples and 274 seronegative samples as demonstrated by MN assay. All samples were anonymized before the study and were used for MERS-CoV Abs testing based on ethical approval obtained from the Unit of Biomedical Ethics in King Abdulaziz University Hospital.

2.3. MN assay

Microneutralization assay was performed as previously described (Al-Amri et al., 2017) in the BSL-3 facility at the Special Infectious Agents Unit (SIAU), King Fahd Medical Research Center, King Abdulaziz University. Briefly, heat inactivated serum samples were tested to determine the highest dilution that inhibits cytopathic effect (CPE) of a 100 $TCID_{50}$ of MERS-CoV/Hu/Taif/SA/2015 in Vero E6 cells. Virus was mixed with an equal volume of serially diluted serum samples starting from 1:5 dilution in 96-well plates and incubated on confluent Vero E6 cells monolayers in 96-well plates for 3 days at 37 °C in a 5% CO_2 incubator. Each serum dilution was tested in quadruplicate. Neutralizing Ab titer (nAb) was determined as the reciprocal of the highest dilution that completely prevent CPE in all wells, and MN_{100} titer of ≥1:10 was considered as positive. Positive and negative controls were always included.

2.4. Recombinant MERS-CoV proteins

Recombinant MERS-CoV spike (S) proteins including ectodomain S protein (amino acids 1–1297) and S1 subunit (amino acids 1–725) tagged with histidine tag were purchased from Sino Biological. Recombinant MERS-CoV N coding sequence from MERS-CoV-Jeddah-human-1 (accession number KF958702) isolate was cloned into pQE2 prokaryotic expression plasmid. Viral RNA was extracted and coding cDNA was PCR amplified by RT-PCR using the following forward primer 5′-GAT CGC GGC CGC GAT GGC ATC CCC TGC TGC ACC TCG TGC TG -3′ and reverse primer 5′- GAT CGG TAC CTT AAT CAG TGT TAA CAT CAA TCA TTG GAC C -3′ to introduce *NotI* and *KpnI* restriction sites at the 5′ and 3′ ends, respectively. Amplified product was purified, digested and ligated into pQE2 vector to generate pQE2-N in which the recombinant protein was tagged at the N-terminus with six histidine residues (rN-6xHis). Cloning was confirmed by restriction digestion and sequencing. Recombinant MERS-CoV N protein was expressed and purified from *E. coli BL21 (DE3)* cells using a nickel-nitrilotriacetic acid (Ni-NTA) column according to the manufacturer's protocol. Positive fractions were pooled and stored at −80 °C until use. All proteins were confirmed by Western blot using anti-His tag Abs, MERS-CoV seropositive and seronegative human serum samples.

2.5. Indirect ELISA

Immulon 2 HB 96-well plates (Thermo Scientific, Rochester, NY) were coated with 100 µl of MERS-CoV rS1 (2 µg/ml), rS (2 µg/ml), or rN (4 µg/ml) proteins diluted in phosphate buffered saline (PBS) for overnight at 4 °C. Plates were then washed with PBS with tween-20 (0.05%) (PBS-T), and blocked with 200 µl PBS-T with 5% skim milk for 1 h at 37 °C. After washing, plates were incubated with 100 µl/well of each serum samples diluted at 1:400 in blocking buffer for 1 h at 37 °C. Plates were washed and incubated with peroxidase-conjugated sheep anti-human IgG (Amersham ECL, Pittsburgh, PA) at 1:2000 dilution for 1 h at 37 °C. After 6 washes, Tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD) was added to each well and incubated for 30 min, and colorimetric reaction was stopped with TMB BlueSTOP Solution (KPL, Gaithersburg, MD). Absorbance was measured spectrophotometrically at 650 nm. All serum samples were tested in duplicates and samples with an optical density above the cut-off values were considered positive. The preliminary cut-off values for each ELISA assay were calculated as the mean of the negative serum OD values +3 standard deviation (SD) from the 100 known seronegative serum samples at 1:400 dilution.

2.6. Statistical analyses and calculations

The sensitivity of the developed ELISAs was calculated as (the number of true positive samples / the total number of true positive and false negative samples) × 100. The specificity was calculated as (the number of true negative samples / the total number of true negative and false positive samples) × 100. Agreement was calculated as (the

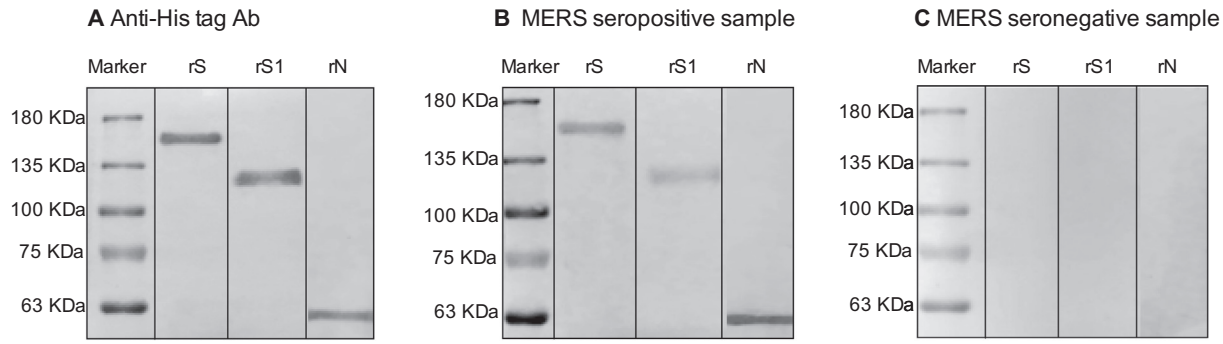

Fig. 1. MERS-CoV recombinant proteins. (A) Recombinant MERS-CoV proteins were detected by Western blot using (A) anti-His tag Abs, (B) known seropositive human serum sample or (C) known seronegative human serum sample. All experiment showed protein bands with expected sizes.

total number of true positive and negative samples / the total number of samples) × 100, and evaluated with kappa values. Receiver operating characteristic (ROC) analysis was calculated using GraphPad Prism software. ROC analysis was performed to determine the relative sensitivity and specificity of each ELISA using MN results as reference test. Univariate analysis using Spearman's correlation analysis between all proposed ELISAs and MN and between ELISAs amongst each other was done using SPSS (IMB, version 22).

3. Results

3.1. MERS-CoV recombinant proteins

The N encoding gene was RT-PCR amplified from MERS-CoV genomic RNA and cloned into prokaryotic expression vector with a histidine tag. Recombinant N Protein was then induced and expressed upon induction with IPTG, and purified on Ni-NTA affinity chromatography column. As shown in Fig. 1A, MERS-CoV rN, rS1 and rS protein bands with expected sizes (~46KDa, ~110KDa and ~155KDa, respectively) were detected with anti-His tag Abs. Western blot with seropositive serum from MERS patient further confirmed the identity of these protein suggesting similar antigenicity to native proteins of MERS-CoV (Fig. 1B). As expected, control MERS-CoV seronegative serum did not react with any of the recombinant proteins (Fig. 1C).

3.2. Development of ELISA based on MERS-CoV recombinant proteins

Three different ELISAs were developed using MERS-CoV rN, rS1 and rS proteins as coating antigens. The optimal working concentrations of each antigen, and serum dilution were determined using checkerboard titration where the highest OD ratio values of positive to negative samples (P/N) were obtained (data not shown). The concentrations of each coating antigen ranged from 0.5 to 8 μg/ml and optimum concentrations were found to be 4 μg/ml for rN and 2 μg/ml for rS1 and rS. Similarly, serum dilution ranged from 1:100 to 1:800 and appropriate serum dilution was found to be 1:400 for all three assays. Using a 100 serum samples from healthy donors that were serologically negative for MERS-CoV, preliminary cut-off values (mean + 3 SD) were found to be 0.30 (mean = 0.13, SD = 0.06) for rN-ELISA, 0.26 (mean = 0.11, SD = 0.05) for rS1-ELISA, and 0.34 (mean = 0.11, SD = 0.08) for rS-ELISA. While all the samples were below the cut-off in the rN-ELISA (Fig. 2A), three and two samples of the 100 samples were above the cut-off values for rS1-ELISA and rS-ELISA (Fig. 2B and C), respectively, with OD values of 0.28 in the rS1-ELISA for the three samples and 0.37 and 0.53 for the two samples in the rS-ELISA.

3.3. Validation of the developed ELISAs

Of the total 353 human serum samples collected from high-risk groups between 2014 and 2017, 79 (22.38%) samples had a MN_{100} titer of 1:10 or higher, and 274 (77.62%) samples had no neutralizing activity (< 1:10) against MERS-CoV and were considered serologically negative by MN assay (Table 1). Screening these samples with the developed ELISAs showed that all the 79 samples were positive by all three ELISAs at the predetermined cut-off values (Fig. 3) without any false negative samples giving a sensitivity of 100% for all three ELISAs. On the other hand, in the MN-seronegative cohort, 113, 28 and 34 individuals were positive when tested by rN-ELISA, rS1-ELISA and rS-Elisa, respectively. These results showed that rS1-ELISA has the highest specificity (89.78%) with good agreement (92.02%, Kappa = 0.797) with MN assay, followed by rS-ELISA which showed a specificity of 87.59% and good overall agreement (90.37%, Kappa = 0.760) with MN assay. The lowest specificity (58.76%) with fair agreement (67.99%, Kappa = 0.389) with MN assay was observed with rN-ELISA.

However, preliminary cut-off values were determined using samples from low-risk group and may not reflect the actual sensitivity and specificity of the assay. Therefore, we next performed ROC analysis to accurately determine the sensitivity, specificity and the cut-off values of the developed assays using samples from both control and high-risk groups. As shown in Fig. 4, the sensitivity of both rS1-ELISA and rS-ELISA remained ≥90% across a wide range of OD values (0.00–0.4 for rS1-ELISA and 0.00–0.48 for S-ELISA), compared to rN-ELISA where sensitivity decreased below 90% when OD value reaches 0.33. Similarly, the specificity of rS1-ELISA and rS-ELISA increased to ≥90% when the OD values were ≥ 0.23 and ≥ 0.31, respectively, in comparison to rN-ELISA which showed a specificity of ≥90% only when the OD value exceeded 0.47. These results suggest that better cut-off values can range between 0.23–0.4 and 0.31–0.48 for rS1-ELISA and rS-ELISA, respectively, to give > 90% sensitivity and specificity. The lowest number of false positive and negative results could be observed at the intersection of the sensitivity and specificity curves. Thus, a more reliable cut-off value of 0.34 for rS1-ELISA could result in 94.9% sensitivity, 95.2% specificity and 95.1% agreement. Similarly, 0.40 cut-off value for rS-ELISA could result in 92.4% sensitivity, 93.3% specificity and 93.2% agreement.

ROC analysis showed low accuracy of rN-ELISA as the area the under curve (AUC) was 0.891 ± 0.020 (95% confidence interval [CI] 0.861 to 0.921). On the other hand, AUC of rS1-ELISA and rS-ELISA were 0.987 ± 0.004 (95% CI 0.979 to 0.995) and 0.985 ± 0.004 (95% CI 0.976 to 0.993), respectively, further suggesting that both rS1-ELISA and rS-ELISA have higher accuracy compared to rN-ELISA (Fig. 5). Testing inter-assay (within plates) and intra-assay (between plates) variability showed very minimal variation in obtained OD values, < 10% (data not shown), suggesting high reproducibility of all assays. Univariate analysis using Spearman's correlation showed with very high significance a strong correlation between MN_{100} titer and OD values of rS1- and S-ELISA ($r = 0.643$, $p < .001$, and $r = 0.640$, $p < .001$ respectively) and a moderately strong correlation between MN_{100} titer and rN-ELISA ($r = 0.517$, $p < .001$). Furthermore, ELISA OD values are very strongly correlated with very high significance

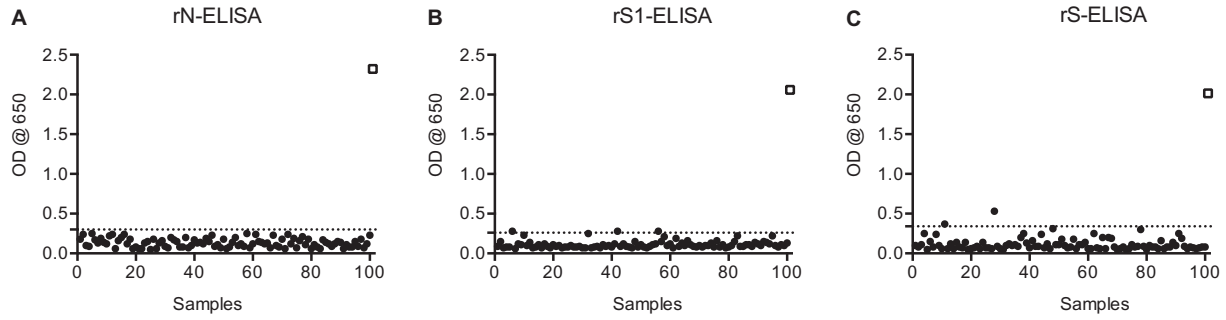

Fig. 2. Cut-off values for the developed ELISAs. A 100 serum samples serologically negative for MERS-CoV from healthy blood donors were used to determine the cut-off values for (A) rN-ELISA, (B) rS1-ELISA and (C) rS-ELISA. Cut-off values were calculated as mean + 3SD. The empty square is a serologically positive control sample. The dotted line represents the cut-off of the assay.

Table 1
Distribution of test samples.

MERS-CoV MN_{100} titer	n (%)
1:640	1 (0.28)
1:320	4 (1.13)
1:160	3 (0.85)
1:80	7 (1.98)
1:40	16 (4.53)
1:20	25 (7.08)
1:10	23 (6.52)
< 1:10	274 (77.62)
Total	353

amongst each other as follows: rN- and rS1-ELISA ($r = 0.895$, $p < .001$), rN- and rS-ELISA ($r = 0.891$, $p < .001$), and rS1- and rS-ELISA ($r = 0.844$, $p < .001$).

4. Discussion

Direct virus detection methods are limited by the need for proper sampling during virus shedding periods. On the contrary, anti-MERS-CoV Abs can be detected over an extended period of time, thus validated serological assays could represent a valuable tool in diagnosis as well as epidemiological and surveillance studies. Furthermore, levels of anti-MERS-CoV Abs have been shown to be associated with disease severity and viral loads, suggesting that they could be utilized as prognostic markers for disease outcomes (Poissy et al., 2014; Corman et al., 2016; Choe et al., 2017; Zhao et al., 2017). Current gold standard virus neutralization assays (MN and PRNT) or the alternative ppNT assay are sensitive, specific and have excellent correlation with each other (Park et al., 2015). However, they require skilled personnel working with live virus in high containment laboratories or availability of specialized equipment and continuous source of pseudoviruses in the case of ppNT assay. Other assays such as IFA and protein microarrays are expensive, time-consuming, less sensitive, and require trained technical staff and specialized equipment. These limitations render such assays not suitable for large-scale or high-throughput screening and testing especially in MERS-CoV endemic regions where most of these requirements are limited or not available. Therefore, we aimed to develop, compare and validate different in house ELISAs based on MERS-CoV N, S1 and S recombinant proteins using large number of well characterized human samples to provide cheap and easy tool for serological testing in resource-limited regions.

While our data showed that all the three developed ELISAs have a 100% sensitivity using the preliminary cut-off values, the specificity of these three assays were below 90%. Nevertheless, ROC analysis of data obtained from both sample groups showed that sensitivity of rS1 and rS ELISAs was maintained at ≥ 90% across a wider range of OD values (< 0.40 and < 0.48 for rS1-ELISA and S-ELISA, respectively) compared to rN-ELISA (sensitivity decreased below 90% at ≥0.33 OD value). Also, the specificity of rS1 and rS ELISAs reached 90% or higher at OD values of ≥ 0.23 and ≥ 0.31 for rS1-ELISA and S-ELISA, respectively, giving bigger dynamic range for these two assays (0.23–0.4 and 0.31–0.48 for rS1-ELISA and rS-ELISA, respectively) compared to rN-ELISA where high specificity (≥90%) resulted in huge loss of sensitivity. Additionally, both rS1 and rS ELISAs showed higher accuracy, better correlation and good overall agreement with MN assay in comparison to rN-ELISA. These data suggest that rS1-ELISA followed by rS-ELISA are more sensitive, specific and accurate than rN-ELISA when benchmarked to results determined from MN-assays. Furthermore, as shown by ROC analyses more reliable cut-off values of 0.34 and 0.40 for rS1-ELISA and rS-ELISA, respectively, could be used in epidemiological and surveillance studies. These cut-off values indicate likelihood of having specific neutralizing antibody titer > 10. However, values that fall between the preliminary cut-off determined from the healthy donors and ones determined using ROC analysis should be considered "indeterminate" and should be validated with other methods if possible.

While we found that rN-ELISA has lower sensitivity compared to

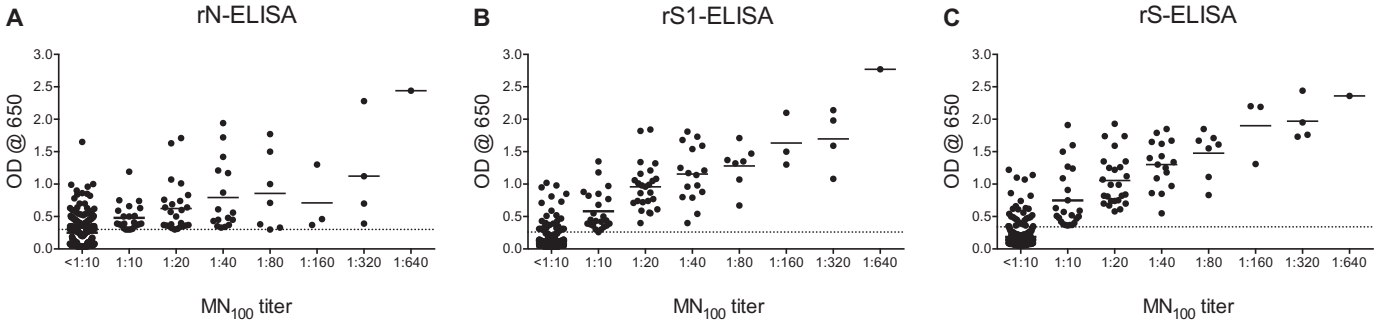

Fig. 3. ELISA results of the serologically negative and positive serum samples. The test results for (A) rN-ELISA, (B) rS1-ELISA and (C) rS-ELISA were plotted as OD value at 650 nm for each sample group based on their MN_{100} titer. The dotted line represents the cut-off of each assay.

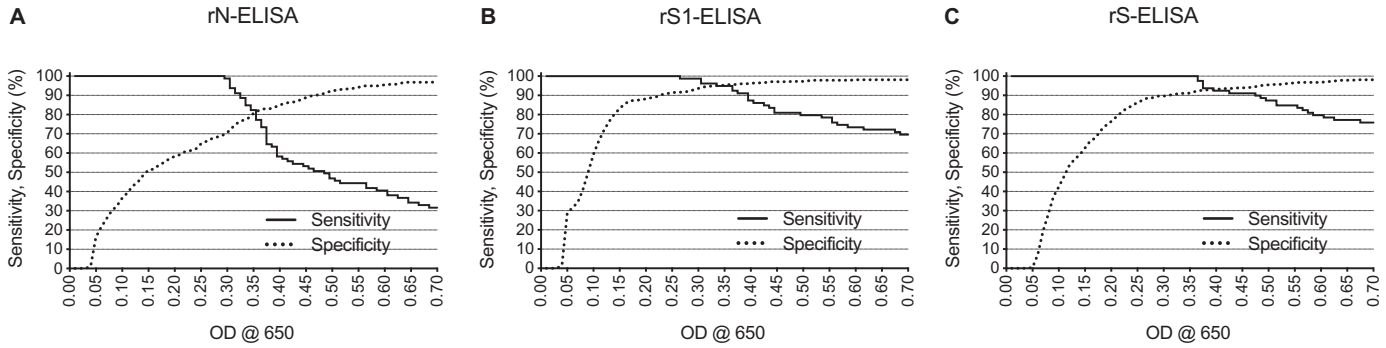

Fig. 4. Relative sensitivity and specificity of the developed ELISAs using Two-Graph-ROC analysis. Sensitivity and specificity of (A) rN-ELISA, (B) rS1-ELISA and (C) rS-ELISA are plotted (y-axis) against OD values (x-axis).

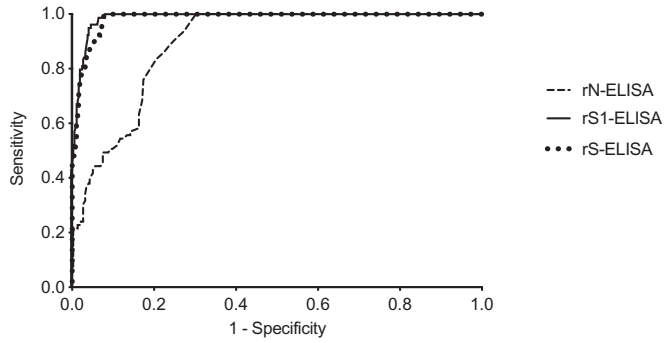

Fig. 5. Receiver operating characteristics (ROC) analysis. ROC analysis was applied to serologically positive vs. negative MERS-CoV samples identified by MN assay at MN_{100} titer of 1:10 for rN-ELISA, rS1-ELISA and rS-ELISA.

both rS1 and rS ELISAs in line with a previous report (Wang et al., 2016), it was recently suggested to have higher sensitivity compared to rS-ELISA across a wide range of OD values (Trivedi et al., 2017). This discrepancy could be explained by the small number of clinical samples especially from high-risk groups used in previous studies which could represent a limiting factor to actually validate these assays. Furthermore, while N protein is more immunogenic and abundant compared to S glycoprotein, and that some patients might have delayed or no development of anti-S Abs (Chen et al., 2015; Trivedi et al., 2017), common antigenic epitopes across the different coronaviruses in N protein could lead to high level of false positivity as we observed in rN-ELISA (Agnihothram et al., 2014; Chen et al., 2015; Trivedi et al., 2017). On the other hand, S1 subunit is associated with less cross-reactivity due higher diversity amongst coronaviruses (Park et al., 2015). In addition, genetically diverse MERS-CoV clades are serologically indistinguishable by neutralization assays which detect nAbs targeting epitopes within the S1 subunit (Hemida et al., 2014; Muth et al., 2015).

Nonetheless, it is noteworthy to underscore that individuals from high-risk group who had positive ELISA results in the three developed assays without detectable nAbs in MN assay could have been exposed to MERS-CoV and mounted a true IgG response but lacked nAbs especially that accumulating body of evidence suggests that Ab responses in mild or asymptomatic MERS cases might be transient (Alshukairi et al., 2016; Drosten et al., 2014). Furthermore, it was recently shown that serological testing by ELISA or MN assay might not capture all exposed or infected individuals as some high-risk individuals could mount virus-specific T cell responses rather than detectable Ab responses (Alshukairi et al., 2016; Alshukairi et al., 2018). It is of note that the 28 false positive samples detected by rS1-ELISA were also positive in rS-ELISA and rN-ELISA, highly suggesting they could be true seropositive samples lacking neutralizing activity. Therefore, we believe future studies are needed to further evaluate these ELISA assays or an ELISA based on pooled antigens in comparison with other methods such as virus-specific T cell response, IFA and neutralization assays.

Several assays have been developed for MRES-CoV serological testing including gold standard neutralization assays which are not practical for field or clinical applications because of the need to use live MERS-CoV in high containment facilities. Furthermore, several groups have already reported development of ELISA based assays for MERS-CoV, but have primarily used these assays for animal model development or testing of samples from camels or other livestock. In the current report, we developed and evaluated three different ELISA-based assays for MERS-CoV serological testing using large number of characterized clinical samples. These assays were found to have variation in their sensitivity, specificity, accuracy, and correlation with MN assay where rS1-ELISA followed by rS-ELISA performed better than rN-ELISA. These developed ELISAs especially those based on rS1 or rS could be used independently or in combination with rN-ELISA in large-scale and high-throughput serological and epidemiological screening as they do not require high containment laboratories and could be adapted by any lab especially that all required reagents are commercially available.

Authors contribution

AMHash conceived and designed the study, AMHass, SIH, OBH, NSA, ANA and EIA collected and provided the samples. SSA, TLA and LAS performed the experiments, AMHash, RYA and AAM analyzed the data, AMHash drafted the manuscript, AMHash, MMA, ANA, AAM and EIA revised the manuscript, all authors reviewed and approved the manuscript.

Conflict of interest

None.

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