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Development and Validation of a Rapid Immunochromatographic Assay for Detection of Middle East Respiratory Syndrome Coronavirus Antigen in Dromedary Camels

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We present here a rapid immunochromatographic assay for the detection of Middle East respiratory syndrome coronavirus (MERS-CoV) antigen in the nasal swabs of dromedary camels. The assay is based on the detection of MERS-CoV nucleocapsid protein in a short time frame using highly selective monoclonal antibodies at room temperature. The relative sensitivity and specificity of the assay were found to be 93.90% and 100%, respectively, compared to that of the UpE and open reading frame 1A (Orf1A) real-time reverse transcriptase PCR (RT-PCR). The results suggest that the assay developed here is a useful tool for the rapid diagnosis and epidemiological surveillance of MERS-CoV infection in dromedary camels.

Middle East respiratory syndrome coronavirus (MERS-CoV) is a newly identified human coronavirus associated with severe pulmonary syndrome and renal failure in infected patients (1). To date, a total of 843 persons in 21 different countries have been infected by the virus, with a resulting 37.95% mortality rate (2). The current MERS-CoV outbreak investigations suggest that camels are a source of human infections. Nevertheless, the exact route of transmission from camels to humans remains unclear (3).

MERS-CoV is primarily diagnosed using molecular techniques. These include real-time reverse transcriptase PCR (RT-PCR) (4, 5), reverse transcription–loop-mediated isothermal amplification (RT-LAMP) (6) and reverse transcription-recombinase polymerase amplification (RT-RTPA) (7). Moreover, several serological assays have been used to detect MERS-CoV or closely related viruses in seropositive camels. These are protein microarrays (8–10), a recombinant spike immunofluorescent assay (11, 12), indirect enzyme-linked immunosorbent assay (ELISA) (13), microneutralization, and spike pseudoparticle neutralization (14). However, none of the serological tests have provided proof of the precise presence of MERS-CoV in camels.

Molecular tests are relatively expensive, not available in all laboratories, and are mainly used for confirmatory purposes. For the purpose of screening of large numbers of animals in a short period of time, molecular tests are considered impractical; therefore, a rapid, cheap, sensitive, and specific test is needed for the diagnosis of MERS-CoV in camels. Here, we report the development and validation of an immunochromatographic assay (ICA) for the rapid qualitative detection of MERS-CoV antigen in dromedary camels. The assay is based on the detection of MERS-CoV nucleocapsid protein by highly selective monoclonal antibodies.

MATERIALS AND METHODS

This study was carried out in two phases during the period of August to October 2014. In the first phase, the ICA was developed at the BioNote laboratory (South Korea). In the second phase, the performance and validation of the ICA were carried out at the veterinary laboratories of the Abu Dhabi Food Control Authority (United Arab Emirates).

Peptides and monoclonal antibody synthesis. At first, the hydrophilic regions of the nucleocapsid gene of MERS-CoV were analyzed by MegAlign. Five peptides named P1 (NLSRGRGRNPKPRAAPNNT) (amino acids [aa] 22 to ~40), P2 (DGATDAPSTFGTRNPNNDSAI) (aa 126 to ~146), P3 (GTGGNSQSSSRASSVSRNSSRSSSQGSRSGNSTRGT SPG) (aa 164 to ~202), P4 (QPKVITKKDAAAAKNKMRHKRTSTKS) (aa 234 to ~259), and P5 (TQRTRTRPSVQPGPMIDV) (aa 393 to ~410) were then selected, synthesized, and conjugated to bovine serum albumin (BSA) by Peptron Corp. (South Korea). The conjugated peptides were used as injectable immunogens for BALB/c mice (6 to 8 weeks old). Three booster injections consisting of 200 μl of the same synthetic peptide (2 mg/ml BSA) antigen emulsified with Freund's adjuvant (Sigma) were given intraperitoneally every 2 weeks. Three days later, after the third injection, each immunized mouse was euthanized, and the spleen cells were isolated. Hybridomas were produced by fusing spleen cells with the mouse myeloma cell line Sp2/0 by polyethylene glycol (15). The hybridomas were then selected in hypoxanthine-aminopterin-thymidine–armitage (HAT-ARMI) medium (Gibco)–10% fetal calf serum (FCS). The antibody screening cells were further cloned using the limiting dilution method. The culture supernatants of the cloned cells were then collected as a source of monoclonal antibodies, and five cloned hybridomas were selected for an immunoblotting assay. All animals used in the development of antibodies were approved by the National Veterinary Research and Quarantine Service animal ethics committee (approval no. BN14-02).

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Preparation of MERS-CoV recombinant nucleocapsid protein. The MERS-CoV recombinant nucleocapsid protein (NC) amino acids 10 to 413 were produced to be used as an internal control. The nucleotides and amino acids were obtained from the National center of Biotechnology Information (http://www.ncbi.nlm.nih.gov) .Further, MERS-CoV genomic DNA was synthesized by BioNeer, Inc (South Korea) and used as a template in the PCRs. The oligonucleotide primers MERS-CoV F (5′-GCTAGCCGA TCGGTTTCCTTTGCCGATAAC-3′) and MERS-CoV R (5′-AAGCTTCTA ATCAGTGTTAACATC-3′) were designed with reference to GenBank accession no. KJ556336. The cycling profile of PCR consisted of a first denaturation step at 94°C for 5 min, followed by 25 cycles at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were then cloned into the NHeI/HindIII restriction site of the pET-28a-TEV vector, which enabled a six-residue histidine tag to be fused to the protein. *Escherichia coli* electrocompetent cells (strain BL21) were then transformed with the recombinant plasmids. To achieve protein expression, a single colony was grown in 2× YT medium (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl) containing 0.05 mg/ml kanamycin for 16 h. This culture was then inoculated in 1 liter of fresh 2× YT medium with kanamycin, maintained under the same conditions above, and induced with 0.05 mM IPTG (isopropyl-β-D-thiogalactopyranoside). When the culture reached an optical density at 600 nm of 0.6, protein expression was carried out for 4 h at 37°C. The protein was then purified using a 5-ml HisTrap column attached to an Akta prime chromatography system (GE Healthcare Life Sciences), eluted with 0.5 M imidazole (20 mM sodium phosphate, 0.5 M NaCl, 0.05 M imidazole, 8 M urea), and stored.

Establishment and assembly of the ICA. The monoclonal anti-MERS-CoV P1 antibody to the MERS-CoV nucleocapsid was coated on a specific area on the nitrocellulose membrane (test line [T]), while goat anti-mouse IgG was coated on another specific area on the same membrane (control line [C]), at a concentration of 1 mg/ml for each. The membrane was then dried and kept tightly sealed at room temperature. A trisodium citrate reduction method was used to produce colloidal gold with a diameter of 25 nm. A solution of 1 ml of 1% chloroauric acid (Sigma, St. Louis, MO) was diluted to a final concentration of 0.01% with 100 ml of deionized double-distilled water and then heated to boiling in a flask with a condensing unit. A volume of 2 ml of 1% sodium citrate solution was then added, the mixture was heated until the solution showed a wine red color, and it was kept in a brown bottle at 4°C after cooling.

To produce the test conjugate, 10 ml of the colloidal gold was mixed with the monoclonal anti-MERS-CoV P3 after the pH was adjusted to 5.4 with 0.1 mol/liter NaOH. Five minutes later, 1 ml of 5% bovine serum albumin (BSA) was added. The mixture was then centrifuged at 10,000 rpm and 4°C for 1 h. The supernatant was then decanted and the pellet dissolved in 10 ml of Tris-buffered saline (TBS) buffer. After a second centrifugation at the same speed and temperature, the pellet was again dissolved in 1 ml of TBS buffer.

The assay strips were prepared by laminating the nitrocellulose membrane, a colloidal gold-conjugated glass fiber, and an absorbent paper by a polyvinyl chloride self-adhesive floor. The strips were cut by a slitter and kept tightly sealed (Fig. 1).

Assay procedure. The camel nasal swabs were transferred from the field to the laboratory in a universal transport medium (TM) (Copan, Italy) within 24 h after collection and stored at −80°C until testing.

The test strips and samples were kept at room temperature prior to testing. A total of 100 μl of the nasal swab transport medium was transferred into a test tube containing 100 μl of added assay diluents (50 mM Tris [pH 8.5] [catalog no. 0862; Amresco], 10 mM NaCl [catalog no. S7653; Sigma], 0.1% Tween 20 [catalog no. P1379; Sigma], 1 ppm ProClin 300 [catalog no. 48914-U; Sigma]). The test strip was then placed into the test tube, with the arrows on the strip pointing down, and the results were read after 15 min. The test was considered negative when only the control (C) line appeared, whereas it was considered positive when both the test line (T) and the control line (C) appeared. In the absence of the control line (C), the test was considered invalid (Fig. 1).

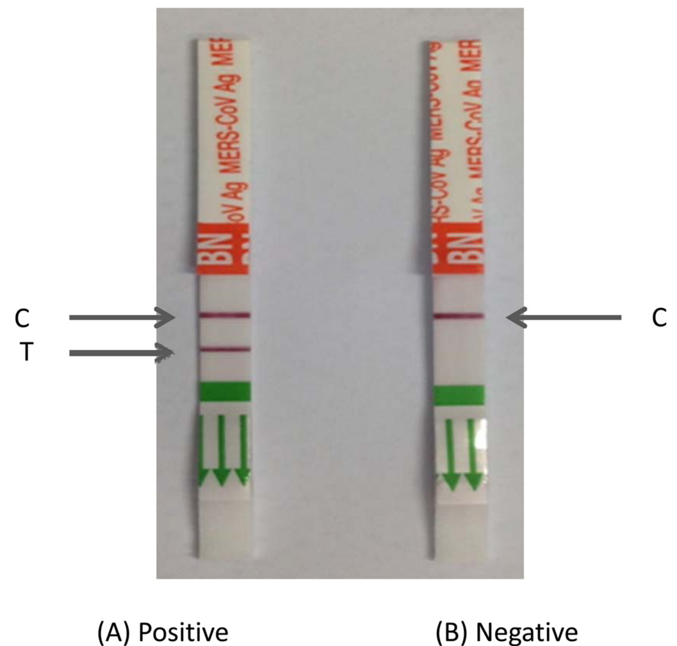

FIG 1 ICA procedure. C, control line; T, test line. Shown are a positive result (A) and a negative result (B).

Personal protective equipment, including N95 masks, goggles, disposable gowns, gloves, and head covers were used during sample collection, shipment, and testing.

Validation of the ICA. To evaluate the specificity of the ICA, the assay was first used to screen coronaviruses of other animals, including bovine coronavirus (BVC) field and vaccine strains obtained from the Korea Veterinary Culture Collection and Green Cross Veterinary Products, respectively, canine coronavirus (CCV), and feline coronavirus (FCV) obtained from the ATCC, together with 18 nasal swabs from camels used as negative control. The camel nasal swabs were from Advanced Scientific Group (Abu Dhabi, United Arab Emirates), where the camels were kept healthy for breeding. These camels were also negative with the UpE and Orf1A real-time RT-PCR.

To obtain the detection limit of the ICA, the MERS-CoV recombinant NC protein was diluted into 2-fold steps from 1,600 ng/ml to 0.78 ng/ml and tested with the ICA kit. Furthermore, MERS-CoV-positive camel nasal swabs that previously were confirmed positive by the UpE and Orf1A real-time RT-PCR were diluted in 2-fold steps from 2^1 to 2^{12}. All dilutions were then tested simultaneously by real-time PCR and the ICA kit.

The sensitivity of the ICA was evaluated using RNA from cultured MERS-CoV. Briefly, Vero (African green monkey kidney, ATCC CRL-1586) cells were maintained at the Integrated Research Facility (Frederick, MD) in Dulbecco's modified Eagle's medium (Corning, Inc., Corning, NY) and 10% fetal bovine serum. The cells were plated at a concentration of 4×10^4/well in 96-well plates (catalog no. 3603; Corning). When the cells were at or near confluence, they were infected with the 10-fold serial dilutions of the Jordan strain of MERS-CoV (10^7 50% tissue culture infective dose [$TCID_{50}$]/ml; GenBank accession no. KC776174, MERS-CoV strain Hu/Jordan-N3/2012). RNA from the cultured virus was extracted using the QIAamp viral RNA kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. For the detection of viral RNA, 5 μl of RNA was used in a one-step real-time reverse transcription-PCR UpE assay using the Rotor-Gene probe kit (Qiagen), according to the manufacturer's instructions. Standard dilutions of a virus stock whose titers

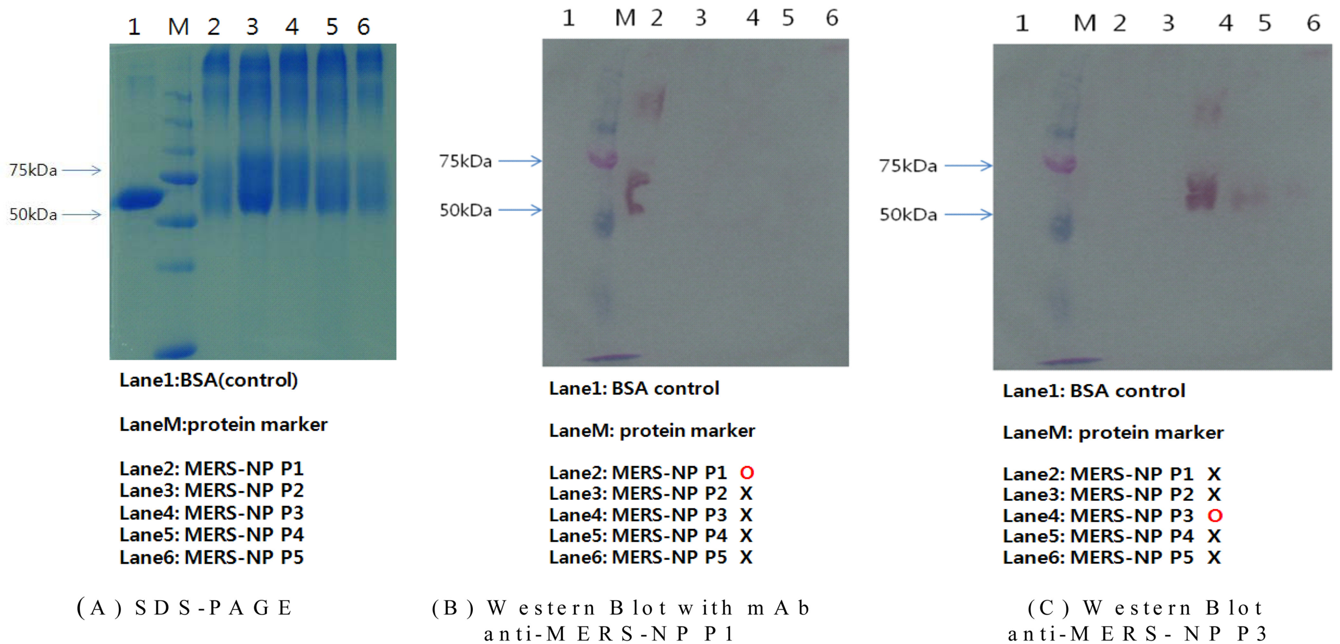

FIG 2 SDS-PAGE and Western blot analysis of 5 synthetic peptides for the nucleocapsid protein. Lane 1, BSA; lane M, protein marker; lane 2, synthetic peptide MERS-NP P1; lane 3, synthetic peptide MERS-NP P2; lane 4, synthetic peptide MERS-NP P3; lane 5, synthetic peptide MERS-NP P4; lane 6, synthetic peptide MERS-NP P5. (A) A 10% SDS-PAGE gel viewed after Coomassie blue staining for 5 synthetic peptides for the nucleocapsid protein. (B) Western blot showing the synthetic peptide MERS-NP P1 detected using monoclonal anti-MERS P1 (#15) and optimized concentration of the antibody (1:5,000 [vol/vol]). O indicates the reactivity of MERS-NP P1 compared to that of the other peptides (X). (C) Western blot showing the synthetic peptide MERS-NP P3 detected using monoclonal anti-MERS P1 (#46) and optimized concentration of the antibody (1:5,000 [vol/vol]). O indicates the reactivity of MERS-NP P3 compared to that of the other peptides (X).

were determined were run in parallel to calculate the $TCID_{50}$ equivalents in the samples. The UpE real-time reverse transcription-PCR was carried out as previously reported (4).

To evaluate the relative specificity and sensitivity and the agreement between the ICA and RT-PCR (as a reference gold standard method), 571 camel nasal swabs were tested concomitantly by the ICA and the UpE and Orf1A real-time RT-PCR. The diagnostic efficacy of the ICA in terms of relative sensitivity and specificity was calculated as described before (16).

The repeatability of the ICA was measured by analyzing 20 replicates of a negative nasal swab sample and 20 replicates of a positive sample, whereas the reproducibility of the ICA was measured by analyzing 5 positive and 5 negative samples in 3 replicates by two different analysts on two different days.

Thermostability of the ICA. The performance of the rapid MERS-CoV antigen kit was also evaluated under different reaction temperature conditions, including 25°C, 37°C, and 45°C.

RESULTS

Production and identification of monoclonal antibodies. The five selected cloned hybridomas reacted to distinct epitopes and were not identical through the immunoblotting assay (Fig. 2). These were termed monoclonal anti-MERS-CoV P1, monoclonal anti-MERS-CoV P2, monoclonal anti-MERS-CoV P3, monoclonal anti-MERS-CoV P4, and monoclonal anti-MERS-CoV P5. Among them, the monoclonal anti-MERS-CoV P1 specific for aa 22 to ~40 and the monoclonal anti-MERS-CoV P3 specific for aa 164 to ~202 were selected as a capture and a detector for the immunochromatographic assay, respectively, as they were reactive with Western blotting and produced the best sensitivity and specificity results.

Generation of the MERS-CoV nucleocapsid protein. Using SDS-PAGE and Western blot analyses, it was shown that the recombinant MERS-CoV nucleoprotein (NP) (44.5 kDa) was successfully expressed and detected with the monoclonal anti-MERS-CoV P3 antibody.

Specificity, sensitivity, repeatability, and reproducibility of the ICA. The ICA specificity was determined using coronaviruses of other animals, including BVC, CCV, and FCV. None of them were detected. Moreover, all 18 negative-control samples were also negative by the ICA, with a resulting specificity of 100%.

The ICA successfully detected MERS-CoV-NC protein at different concentrations. The detection limit of the assay was found to be 1.5 ng/ml. The intensity of the color observed at the test line (T) correlated with the concentration of the recombinant antigen shown on each strip. When the ICA was compared with the UpE and Orf1A real-time RT-PCR on the field sample, the ICA detected MERS-CoV at up to 2^6 dilutions, while the UpE and Orf1A real-time RT-PCR detected MERS-CoV at up to 2^8 dilutions (Table 1).

The sensitivity of the ICA was determined using a dilution range of 10^7 to 10^1 $TCID_{50}$ of cultured MERS-CoV. The UpE real-time PCR showed a sensitivity of up to 10^4 $TCID_{50}$, while the ICA showed a sensitivity of up to 10^5 $TCID_{50}$ (Table 2).

The repeatability of the ICA was found to be >95% when 20 negative and positive replicates of camel nasal swabs sample were tested. In addition, when 5 positive and 5 negative samples in 3 replicates were subjected to the ICA, all the results were the same within each replicate. The durations of time required for the bands to appear on the strips on the test and control lines were the same for all samples. The results also did not differ when the ICA was performed by two different analysts.

TABLE 1 Detection limits of the UpE and Orf1A real-time RT-PCR and the MERs-CoV-ICA on camel nasal swabs

Dilution	PCR result	C_T value[a]	ICA kit result
Original	Positive	27.08	Positive
2^1	Positive	30.78	Positive
2^2	Positive	31.15	Positive
2^3	Positive	31.20	Positive
2^4	Positive	31.34	Positive
2^5	Positive	31.42	Positive
2^6	Positive	32.03	Positive
2^7	Positive	32.22	Negative
2^8	Positive	32.83	Negative
2^9	Negative		Negative
2^{10}	Negative		Negative
2^{11}	Negative		Negative
2^{12}	Negative		Negative

[a] C_T, threshold cycle.

Out of the 571 camel nasal swabs tested, 62 were MERS-CoV positive and 509 were negative by the ICA, whereas 66 were positive and 505 were negative by the UpE and Orf1A real-time RT-PCR (Table 3). Thus, the relative sensitivity and specificity of the ICA compared to those of the real-time RT-PCR were 93.90% and 99.6%, respectively (Table 4).

Stability of the ICA. The ICA performance was stable at 25°C to 37°C, with no change in the color intensity of the test line. However, the reactions at 45°C showed less color intensity and a decreased detection limit of the assay.

DISCUSSION

A definitive diagnosis of MERS-CoV is mainly based on molecular techniques that include real-time RT-PCR (4, 5), as recommended by the WHO, and two newly reported methods (RT-LAMP and RT-RTPA) (6, 7). These methods require skilled personnel, together with specialized instruments and enzymes stored at cool temperatures. Furthermore, the serological assays used in animals have not been able specify that the presence of MERS-CoV antibodies was due to MERS-CoV or other closely related viruses (8). Recently, evidence of camel-to-human transmission of MERS-CoV was documented (3). The absence of clear clinical signs or observed mortalities in MERS-CoV-infected camels represents the potential danger of camels serving as a reservoir for human infections. Therefore, it is important to have a rapid cheap and sensitive test other than the complex molecular techniques used to rapidly diagnose the infected camels. To date, there are no such tests available for the rapid screening of MERS-CoV antigen in camels.

We believe this study to be the first on the development and validation of an ICA capable of detecting MERS-CoV antigen in the nasal swabs of camels. The assay is based on the detection of MERS-CoV nucleocapsid protein by highly selective monoclonal antibodies. In this assay, antigens in the samples first bind to specific gold-labeled monoclonal antibodies (MAbs) on a fiberglass membrane to form an antigen-antibody complex labeled with gold. The complex then moves through a nitrocellulose membrane, where it binds with monoclonal antibody at a test line to form an easily observed band.

The performance of the assay developed here for the detection of MERS-CoV antigen has 100% specificity compared to that of the UpE real-time RT-PCR. On the other hand, the ICA was less sensitive (10^5 $TCID_{50}$) than was the UpE real-time PCR (10^4 $TCID_{50}$). The observed difference between the assay sensitivities might be due to the release of subgenomic RNA after the onset of cytopathogenic effect (CPE) in cell culture, including the UpE target fragment, as previously reported (4).

The evaluations of the assay repeatability and reproducibility indicated that the MERS-CoV antigen detection can be determined with high precision. These results are in accordance with the performances of other reported ICAs (17, 18). Moreover, it is worth noting that the sensitivities and specificities of the World Organisation for Animal Health (OIE)-certified serological diagnostic kits are comparable to the sensitivity and specificity of the ICA (19).

The detection limit of the ICA on the NC protein was 1.5 ng/ml, whereas it was found to be 2^6 compared to that of the real-time RT-PCR (2^8). Generally, rapid screening tests are less sensitive than are confirmatory tests; however, the advantages of using rapid screening tests are the high throughput and rapid turnaround time, without the requirements of sample preparation and the use of special equipment. Therefore, the ICA developed here is considered satisfactory to be used for herd screening against MERS-CoV antigen across international borders, animal markets, and slaughterhouses, followed by a confirmatory test for positive samples. Taking into consideration the biosafety hazard of MERS-

TABLE 2 Sensitivity of the ICA compared to that of the UpE real-time reverse transcription-PCR

$TCID_{50}$	C_T value[a]	Result
10^7	16.86	Positive
10^6	21.61	Positive
10^5	24.07	Moderately positive (faint band)
10^4	27.68	Negative
10^3	ND	Negative
10^2	ND	Negative
10^1	ND	Negative

[a] C_T, threshold cycle; ND, not determined.

TABLE 3 Comparison between performances of the ICA and the RT-PCR

Group	Result	No. with RT-PCR result[a]: POS	NEG	Total no.
ICA	POS	62	2	64
	NEG	4	503	507
Total		66	505	571

[a] POS, positive; NEG, negative.

TABLE 4 Relative sensitivity and specificity of the ICA

Performance parameter	Value	95% CI[a]
Sensitivity (%)	93.90	85.19–98.29
Specificity (%)	99.60	98.57–99.94
Positive likelihood ratio	237.20	59.40–947.12
Negative likelihood ratio	0.06	0.02–0.16
Disease prevalence (%)	11.56	9.05–14.47
Positive predictive value (%)	96.88	89.14–99.53
Negative predictive value (%)	99.21	97.99–99.78

[a] 95% CI, 95% confidence interval.

CoV, it is highly recommended that personal protective equipment (PPE) be used when running the test in the field. It should be noted that the detection limit determined here is an estimate for field samples; therefore, further investigation is required to be performed in order to determine the accurate detection limit of the assay on culture viruses. Based on the fact that the ICA targets the nucleocapsid protein of the virus, which is a conserved region, the assay might in theory be applicable to the medical field for human samples or samples containing other MERS-CoV-related viruses. Therefore, it is worth extending the current assay validation to cover human nasal/nasopharyngeal samples, which in turn might be a very useful tool for the rapid diagnosis of human cases.

In conclusion, a novel ICA for the qualitative detection of MERS-CoV antigen in dromedary camels was successfully developed. The assay was found to be rapid, sensitive, specific, and stable at room temperature. These factors suggest that the assay developed here is fit for its intended purpose and might assist governmental entities in the rapid diagnosis and epidemiological surveillance of MERS-CoV infection in dromedary camels.

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Daesub Song and Gunwoo Ha developed the assay and participated in the lab work. Yassir Eltahir drafted the manuscript and participated in data analysis. Wissam Serhan, Mohammed Yusof, and Farouq Hashem ran the validation experiments. Elsaeid Elsayed, Bahaaeldin Marzoug, and Assem Abdelazim collected the samples. Salama Al Muhairi supervised all the validation experiments and participated in the analysis of the data and the preparation of the final report.

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