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RESEARCH ARTICLE

Countrywide Survey for MERS-Coronavirus Antibodies in Dromedaries and Humans in Pakistan

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Abstract

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is a zoonotic pathogen capable of causing severe respiratory disease in humans. Although dromedary camels are considered as a major reservoir host, the MERS-CoV infection dynamics in camels are not fully understood. Through surveillance in Pakistan, nasal (n = 776) and serum (n = 1050) samples were collected from camels between November 2015 and February 2018. Samples were collected from animal markets, free-roaming herds and abattoirs. An in-house ELISA was developed to detect IgG against MERS-CoV. A total of 794 camels were found seropositive for MERS-CoV. Prevalence increased with the age and the highest seroprevalence was recorded in camels aged > 10 years (81.37%) followed by those aged 3.1–10 years (78.65%) and ≤ 3 years (58.19%). Higher prevalence was observed in female (78.13%) as compared to male (70.70%). Of the camel nasal swabs, 22 were found to be positive by RT-qPCR though with high *Ct* values. Moreover, 2,409 human serum samples were also collected from four provinces of Pakistan during 2016–2017. Among the sampled population, 840 humans were camel herders. Although we found a high rate of MERS-CoV antibody positive dromedaries (75.62%) in Pakistan, no neutralizing antibodies were detected in humans with and without contact to camels.

Keywords Middle East Respiratory Syndrome Coronavirus (MERS-CoV) · Camel · Human · Pakistan

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Introduction

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is a positive sense, single-standard RNA virus of genus *Betacoronavirus*. It is a zoonotic pathogen capable of causing severe respiratory disease in humans. In September 2012, MERS-CoV was first detected in humans and as of May 29, 2018, MERS-CoV infection has been reported from 27 countries with 2,207 laboratory-confirmed cases in humans and at least 787 related deaths (WHO). Majority (80%) of the cases were from Saudi-Arabia (KSA), where a high prevalence of MERS-CoV in dromedary camels and a direct contact with infected camels has been linked to human infections (WHO 2018; Gossner *et al.* 2016; Haagmans *et al.* 2014; Alraddadi *et al.* 2016). Moreover, large hospital linked outbreaks have also been reported from cases imported to other countries (Park *et al.* 2017). Accumulating evidence suggests that camels are reservoir for MERS-CoV. Although camel to human transmission has been documented, most of human infections are due to human to human transmission especially in healthcare settings (Park *et al.* 2017).

In a geographically comprehensive study from KSA, MERS-CoV antibodies were detected in approximately 0.15% of the sampled human population, depicting sporadic infections without severe disease (Müller *et al.* 2015). A significantly higher seroprevalence was observed in slaughterhouse workers and camel shepherds (Müller *et al.* 2015). High prevalence of MERS-CoV antibodies has been reported in camels from different countries of Arabian Peninsula (Saudi-Arabia, United Arab Emirates, Qatar, and Oman) and of Africa (Kenya, Sudan, Nigeria, Burkina Faso, Ethiopia and Morocco) (Gossner *et al.* 2016; Wernery *et al.* 2015; Haagmans *et al.* 2014; Reusken *et al.* 2013; Munyua *et al.* 2017; Müller *et al.* 2014; Chu *et al.* 2018). Presence of MERS-CoV neutralizing antibodies in archived camel sera from 1983 suggests long-term circulation of virus among camel population (Müller *et al.* 2014). However, recent studies from Australia, China and Kazakhstan suggest no evidence of MERS-CoV infection in dromedary and Bactrian camels (Crameri *et al.* 2015; Liu *et al.* 2015; Miguel *et al.* 2016).

A small-scale study from Punjab, Pakistan has reported a high percentage of up to 39.5% of dromedaries having neutralizing antibodies against MERS-CoV (Saqib *et al.* 2017). This is contrasted by the lack of reported human MERS-CoV infection in Pakistan. In view of uncertain disease prevalence, limited capacity for routine surveillance and considerably large human and dromedary population, conducting a countrywide cross-sectional study for MERS-CoV infections in dromedaries and human is of interest for global public health agencies. The aim of this study was to determine the country-wide prevalence of MERS-CoV in camel and human population of Pakistan.

Materials and Methods

Study Locales and Sampling

This cross-sectional study was designed to determine the prevalence of MERS-CoV in camel and human population of Pakistan. For camel sampling, sites were chosen based on dromedary populations and the presence of veterinary clinics (Fig. 1). During 2015–2018, 776 camel nasal swabs were collected. After collection, swabs were placed into tubes containing RNAlater® (Ambion, Austin, USA) and stored at − 80 °C. A total of 1050 dromedary sera were also collected using blood collection system (Becton–Dickinson Co, San Jose, USA). Before taking blood samples the clinical parameters including rectal temperature, body condition and symptoms of any disease were also recorded.

For human sampling, sites were randomly chosen to represent rural, peri-urban and urban areas of Pakistan. Few sampling sites in rural areas were purposively chosen due to the abundance of camel population in those areas. Information related to demographics, exposure to animals and animal products was also recorded. Human serum samples were obtained from hospitals, primary health care centres and blood banks from people older than 15 years of age.

Cloning, Expression and Purification of S-Tagged Receptor-Binding Domain (RBD) of MERS-CoV

A codon-optimized 720-bp RBD- region in the *S* gene of MERS-CoV (accession number: JX869059), which corresponds to aa 367–606 in the spike protein was synthesized at Sangon Biotech (Sangon Biotech, Shanghai, China). The synthesized sequence was cloned into *Xho* I and *Hin*d III sites of mammalian expression vector pCAGGS-S-tag. The protein was expressed in HEK293T cells. Briefly, cells were seeded into large culture dishes (Φ = 15 cm) and grown to 80% confluence. HEK293T cells were transfected with pCAGGS-MERS-CoV-RBD plasmid using lipofectamine 3000 (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Six hours post-transfection, culture medium was replaced with FreeStyleTM 293 Expression Medium (Gibco, Grand Island, USA) and cells were incubated for 48 h at 37 °C, then cell culture supernatant was collected. The MERS-CoV-RBD-S fusion protein was purified using S-protein Agarose (Novagen, Madison, USA) following manufacturer's protocol and was concentrated to 1 mg/mL by Amicon Ultra-4 Centrifugal

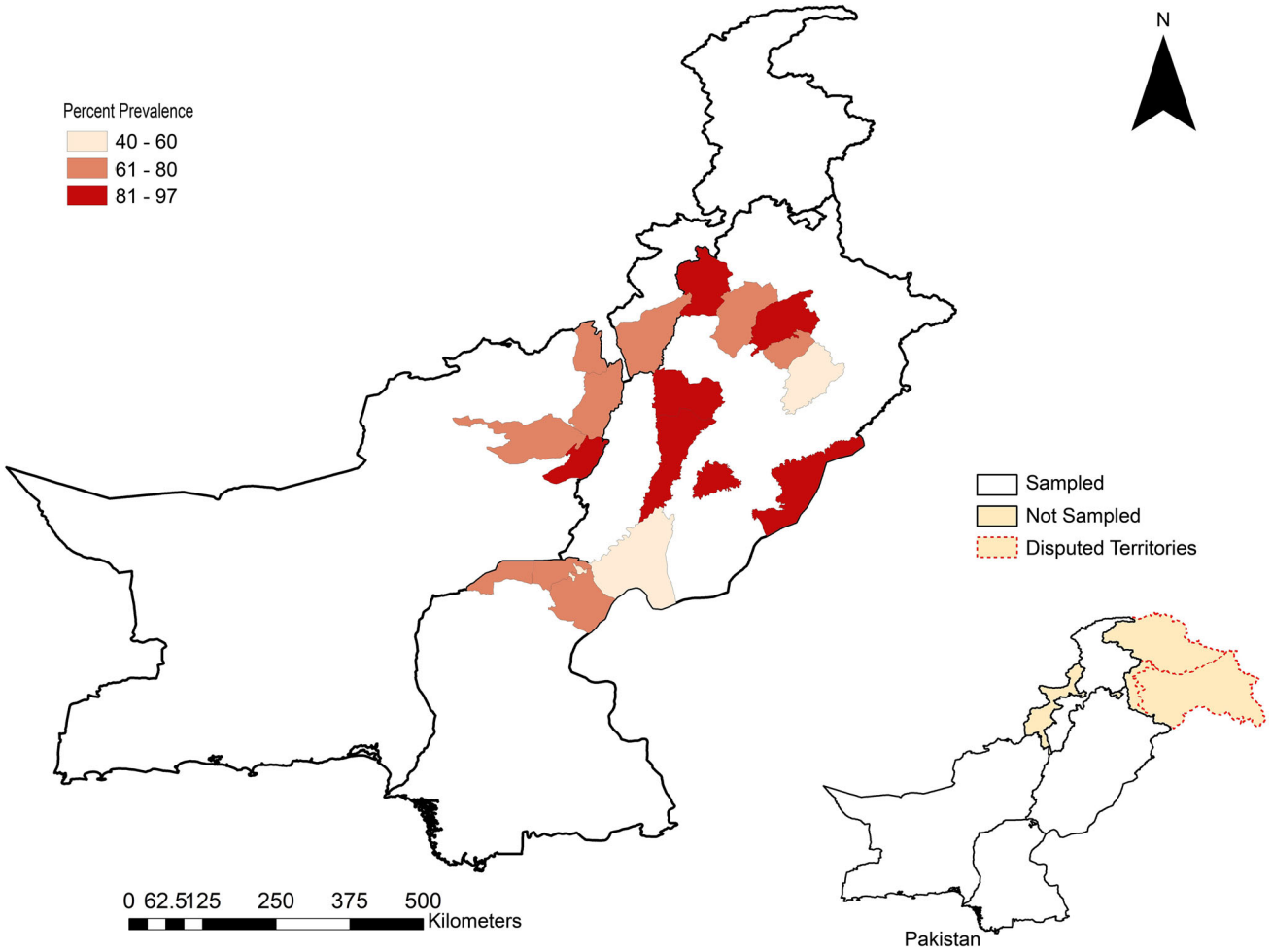

Fig. 1 Map of Pakistan showing seroprevalence of MERS-CoV in camels by districts. Inset is a map of Pakistan showing the boundaries of areas sampled, not sampled and disputed territories.

Filters (Ultracel-3 K; Millipore, Bedford, USA) after centrifuge at 4000 g. The purified protein was analysed by SDS-PAGE and Western blot (Fig. 2).

Serological Testing

For serology, a two-step approach as recommended by WHO for the detection of antibody against MERS-CoV was used. An in-house anti-MERS-CoV IgG rELISA kit was developed. Briefly, MaxiSorp Nunc-immuno 96 well ELISA plates were coated (100 ng/well) overnight with recombinant RBD protein of MERS-CoV. Camel sera were applied at 1:20 dilution for 1 h at 37 °C. An anti-camel IgG-HRP conjugated monoclonal antibody (Kyab Biotech Co.Ltd, Wuhan, China) was used at a dilution of 1:2000. The OD value (450–630) was calculated. On the basis of virus neutralization test, a cut-off value of 0.15 was set for camel sera. To confirm assay sensitivity, a total of 153

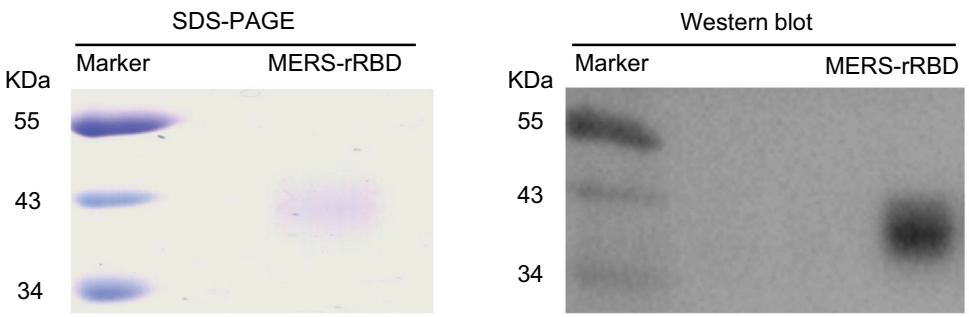

Fig. 2 Expression of MERS-CoV RBD (MERS-rRBD) protein in HEK293T cells analysed by SDS-PAGE and Western blot analysis.

MERS IgG positive samples by commercial ELISA (Euroimmun, Lubeck, Germany) and neutralization assay from a previous study were utilized (Saqib *et al.* 2017). To calculate the specificity, 100 MERS IgG negative samples by ELISA (Euroimmun, Lubeck, Germany) and neutralization assay were tested. Similar to commercially available ELISA (Euroimmun, Lubeck, Germany), in-house ELISA was found to be 100% sensitive and specific.

For human serology, sera samples were applied at 1:20 dilution for 1 h at 37 °C. An anti-human IgG-HPR conjugated monoclonal antibody (Kyab Biotech Co.Ltd, Wuhan, China) was used at a dilution of 1:200000. The samples with optical density ratio $\geq$ 0.2, which is as high as three times the ratio of the mean value of all tested human serum samples without known exposure to camels, were further tested with a commercially available ELISA kit (Euroimmun, Lubeck, Germany).

ELISA positive samples were subjected to microneutralization assay as described previously (Perera *et al.* 2013). Briefly, sera were heat-inactivated (56 °C for 30 min) and then diluted to 1:20. The sera dilutions were then mixed with equal volumes of 100 $TCID_{50}$ of MERS-CoV. After 1 h of incubation at 37 °C, 50 µL of the virus-serum mixture was added in quadruplicate to Vero cell monolayers, in 96-well microtiter plates. After 1 h of adsorption, the media was removed and cells were washed with DMEM. An additional 100 µl of culture medium (DMEM + 2% FBS) was added to each well and the plates were incubated for three more days at 37 °C in 5% CO_2 in a humidified incubator. Cytopathic effect (CPE) was read at three days post infection. Positive and negative control sera were included to validate the assay. Antibody titres of = 1:20 were regarded as positive.

Molecular Testing

The WHO testing algorithm for MERS-CoV was implemented. From camel nasal swabs viral RNA was extracted by using viral RNA extraction kit (Roche, Mannheim, Germany) according to the manufactures instruction. Samples were then tested for the presence of MERS-CoV RNA using N2 RT-qPCR assay as described previously (Lu *et al.* 2014). Samples positive by N2 assay were then confirmed with N3 RT-qPCR assay. Samples positive by N2 and N3 assays were further subjected to nested and hemi-nested PCRs for *S* and *N* genes as described elsewhere (Corman *et al.* 2012; Assiri *et al.* 2016). Sanger sequencing was then performed and ABI files were analysed with Geneious R11. These sequences were submitted to GenBank under accession numbers MH102354 and MH102355.

Statistical Analysis

ELISA positive samples were analysed statistically for the association of age, sex and location by using χ^2 test. A P value < 0.05 was considered significant for all analyses. Univariable analysis was performed and odd ratios along with 95% confidence intervals (CIs) were calculated to determine the association between prevalence and different variables. The analysis was performed in R v3.4.2 using package epicalc v2.15.1.0.

Results

MERS-CoV Infection in Camel Population of Pakistan

A total of 22 samples were found positive by two independent RT-qPCR assays (e.g. N2 and N3 RT-qPCR), though with high *Ct* values > 35. Additionally, from one of these samples, we were able to amplify partial Nucleocapsid and Spike gene segments (GenBank accession nos: MH102354 and MH102355), with a size of 228 bp and 960 bp, respectively. The sequences obtained from the Pakistani camel were identical with several already published sequences obtained from camel as well as humans from the Arabian Peninsula. Unfortunately, likely due to RNA degradation we were unable to recover additional sequences but are confident in the fidelity of the finding. First, viral RNA extraction and RT-qPCR experiments were performed in a laboratory where no previous MERS-CoV work has ever been done. Second, hemi-nested PCR for *N* gene were repeated in two independent laboratories by two different persons yielding identical results. These results indicate active circulation of closely-related or identical strains circulating in Pakistan compared to the Arabian Peninsula.

For serology, 1,050 camel serum samples were collected, out of these 695 (66.19%) were females and 355 (33.81%) were from male camels. The majority of sera were from Punjab Province (57.14%) and semi-nomads (37.10%). The distribution of sera by age, sex, type of herd and sampling location is presented in Table 1. Of 1,050 camel sera tested by ELISA 794 (75.62%) sera were found to be positive by ELISA (Figs. 1, 3). Slightly higher prevalence was observed in camels from Khyber Pakhtunkhwa (KPK) (79.76%, 95% CI 72.72–85.40) compared to Balochistan (77.13%, 95% CI 70.33–82.80), Punjab (74.50%, 95% CI 70.77–77.90) and Sindh (72.34%, 95% CI 61.95–80.83); however, the differences were not significant. Prevalence increased with the age and the highest seroprevalence was recorded in camel aged > 10 years

Table 1 Univariate analyses of Middle East Respiratory Syndrome Coronavirus ELISA-positive camels with their determinants.

Variable	Category	No. positive/No. tested	(%) Prevalence (95% CI)	Odds ratio (95% CI)	*P*-value
Province	Punjab	447/600	74.50 (70.77–77.90)	1.12 (0.66–1.85)	0.432
	Khyber Pakhtunkhwa	134/168	79.76 (72.72–85.40)	1.5 (0.8–2.82)	
	Balochistan	145/188	77.13 (70.33–82.80)	1.29 (0.7–2.35)	
	Sindh	68/94	72.34 (61.95–80.83)	1	
Age	$\leq$ 3 Y	103/177	58.19 (50.54–65.48)	1	$<$ 0.001
	3.1–10 Y	560/712	78.65 (75.42–81.57)	2.64 (1.84–3.8)	
	$>$ 10 Y	131/161	81.37 (74.31–86.89)	3.13 (1.86–5.35)	
Sex	Female	543/695	78.13 (74.83–81.11)	1.48 (1.11–1.98)	$<$ 0.01
	Male	251/355	70.70 (65.62–75.33)	1	
Type of herd	Semi-nomad	278/389	71.46 (66.65–75.85)	1.16 (0.78–1.73)	$<$ 0.001
	Nomad	140/157	89.17 (82.97–93.38)	3.81 (2.06–7.36)	
	Pastoralists	251/321	78.19 (73.19–82.51)	1.66 (1.08–2.55)	
	Sedentary	125/183	68.31 (60.96–74.86)	1	

(81.37%, 95% CI 74.31–86.89) followed by those aged 3.1–10 years (78.65%, 95% CI 75.42–81.57) and $\leq$ 3 years (58.19%, 95% CI 50.54–65.48). The age of camel was the main determinant of prevalence as older animals ($>$ 10 years) were three times more likely to be positive (Odds Ratio 3.13, 95% CI 1.86–5.35) as compared to younger animals ($\leq$ 3 years). Significantly ($P < 0.001$) higher prevalence was observed in females (78.13%, 95% CI 74.83–81.11) as compared to males (70.70%, 95% CI 65.62–75.33). Significantly, higher prevalence was observed in nomadic camels (89.17, 95% CI 82.97–93.38) followed by pastoralists (78.19%, 95% CI 73.19–82.51), semi-nomads (71.46%, 95% CI 66.65–75.85) and sedentary (68.31%, 95% CI 60.96–74.86) (Table 1).

To further verify the ELISA results, 100 ELISA positive and 20 ELISA negative samples were randomly chosen and tested for MERS-CoV neutralizing antibodies by microneutralization assay as described elsewhere (Perera *et al.* 2013). Neutralization assay results were found to be in agreement with the ELISA results, further confirming the sensitivity and specificity of in-house ELISA.

Seroprevalence of MERS-CoV in Human Population of Pakistan

In total, 2,409 human sera samples were collected from four provinces of Pakistan during 2016–2017. Out of 2,409 samples, 1,249 were from females and 1,160 were from males. Most of the sampled population was involved in livestock rearing. Among the sampled population, 840 humans were camel herders.

A total of 91 human sera samples with OD values $\geq$ 0.2, which is as high as three times the ratio of the mean value of all tested human serum samples without known exposure to camels, were further tested with a commercially available ELISA kit (Euroimmun, Lubeck, Germany). Thirty-six samples were found to be positive by commercial ELISA, all having OD values $\geq$ 0.35 (Fig. 4). All the reactive samples were from Punjab Province of Pakistan. Of note, out of these 36 ELISA positives, 30 of them were from camel herders. To further confirm the presence of antibodies in these samples, all 91 sera were tested for MERS-CoV neutralizing antibodies by microneutralization assay as described elsewhere (Perera *et al.* 2013). None of them was positive.

Discussion

Surveillance of emerging zoonotic viruses is a key element of one health program. In the present study, seroprevalence of MERS-CoV in camels and human population in four provinces of Pakistan was estimated. Camels from all four provinces of Pakistan showed evidence of past MERS-CoV infection and 75.62% tested positive for MERS-CoV by ELISA. Our overall seroprevalence data is in agreement with studies from other camel rearing countries (Falzarano *et al.* 2017; Munyua *et al.* 2017). A previous study from Pakistan also described a high seroprevalence of MERS-CoV in camels (Saqib *et al.* 2017). However, that study covered only a limited area of Punjab Province of Pakistan. Our study, not only covered camel rearing areas of Punjab Province but also covered the other three provinces of Pakistan. We observed high seroprevalence of MERS-CoV antibodies in Bahawalnagar district of Pakistan bordering with Rajasthan state of India (Fig. 1), raising the possibility of a spectre of similar level of prevalence, although no study yet been undertaken in Rajasthan, India. Presence of

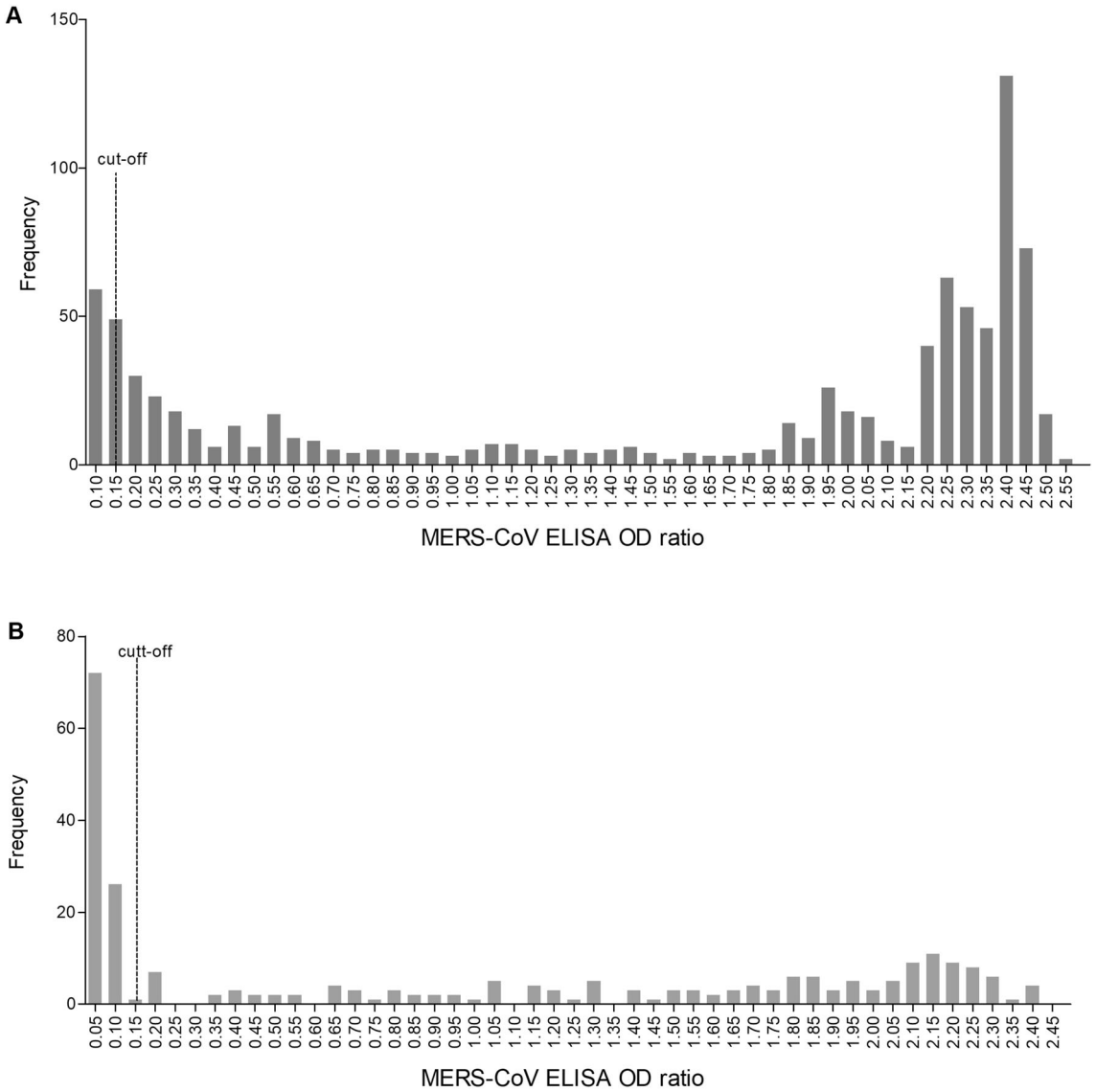

Fig. 3 Histogram displaying the frequency distribution of MERS-CoV IgG ELISA optical density (OD) ratios. **A** ELISA OD ratios for dromedary camels tested in this study. **B** ELISA OD ratios for dromedaries used to determine the sensitivity and specificity. The vertical dashed lines represent the ELISA cut-off values.

IgG-positive animals in KPK and Balochistan Province of Pakistan expands the distribution of MERS-CoV to the westward. Balochistan shares its border with Afghanistan and Iran. Both countries harbour large population of camels. Studies should be carried out in these countries and around border areas to determine the ecology of MERS-CoV in these regions. Significantly higher seroprevalence was observed in old age camels in this study. Similar age-dependent differences have previously been reported (Corman *et al.* 2014). This could be due to regular re-exposure of MERS-CoV after initial infection.

Countrywide high prevalence of MERS-CoV in dromedaries suggests a risk for human exposure similar to the one reported from Arabian Peninsula. Of 2409 samples tested, 36 samples were positive by commercial ELISA, however, none tested positive for neutralizing antibodies against MERS-CoV.

A previous study from KSA has reported 23 times increased risk of being seropositive for MERS-CoV in persons with occupational exposure to camels (Müller *et al.* 2015). Among the tested population of Pakistan, 840 persons were camel herders. These camel herders had very close interactions with the camels including direct handling (i.e. herding, milking and slaughtering) and consumption of unpasteurized milk. All of these local practices of camel herders provide plentiful opportunities for contact with camels and their products. Despite human-camel interaction, no neutralizing antibodies for

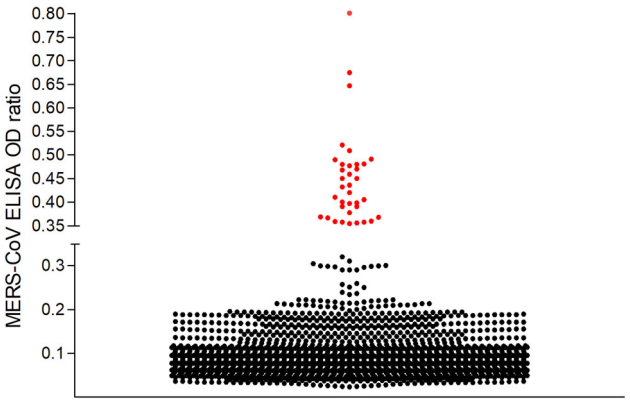

Fig. 4 Scatter dot plot of all human individual optical density (OD) ratios obtained from anti-MERS-CoV in-house ELISA. All samples found to be positive by commercial ELISA (Euroimmun, Lubeck, Germany) are shown in red.

MERS-CoV were found in these human individuals. This may imply no camel-to-human transmission in the studied population in this period. Although camel-human transmission of MERS-CoV is infrequent in the Arabian Peninsula and Africa, however, the absence of neutralization antibodies in camel herding communities was unanticipated. Our finding corroborates with those of previous studies from Kenya that have reported ELISA positive but in most instances neutralization negative human sera (Liljander *et al.* 2016; Munyua *et al.* 2017). It is, therefore, possible that the MERS-CoV strains circulating in Pakistan and Kenya are antigenically different from the strains used in the neutralization assays (Liljander *et al.* 2016; Munyua *et al.* 2017). Moreover, the short life-span of anti-MERS-CoV antibodies, especially in humans with mild disease may explain the absence of detectable neutralizing antibodies (Alshukairi *et al.* 2016). Our results suggest a likely reduced transmissibility of MERS-CoV from camels to humans with the same level of exposure as persons in KSA, though the mechanism is unclear. Albeit we studied a large human population from Pakistan for the past exposure of the MERS-CoV infection, however, owing to potentially waning immunity in subclinical MERS-CoV infection in human, it is possible that this study provided a finite opportunity to make a conclusive statement on exposure status of MERS-CoV in humans in Pakistan. Prospective studies need to be conducted to undertake the factors responsible for the rarity of seropositivity to MERS-CoV in camel herders in Pakistan.

The high seroprevalence of MERS-CoV among young camels (58.19%) indicates recent active circulation of MERS-CoV in camel populations of Pakistan. The active circulation of MERS-CoV in Pakistan is further confirmed by the detection of MERS-CoV in 22 nasal swab samples by N2 and N3 RT-qPCR assays in this study. This continuous circulation of MERS-CoV in camel population of Pakistan poses a possible risk of human transmission to camel herders and other people who have frequent contact with camels or their products. On one hand, active virus circulation and high seroprevalence of MERS-CoV in camel population were observed, whereas, on the other, the absence of human cases in Pakistan poses a germane question regarding the viral epidemiology in different geographic and ecological zones.

To the best of our knowledge, this is first country-wide study on MERS-CoV seroprevalence in camel and human population of Pakistan. Further longitudinal studies among camel herding communities as well as studies on detection and characterization of MERS-CoV strains in camels from Pakistan are warranted.

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Author contributions AZ, MS, MAA, JC, MSS and VMC wrote the manuscript. AZ, MS. MAA, ARS, SK, ZT, HS, UT, MHT, MAQ, MKM, BAK, IDU, IK and MSS participated in the sampling. AZ, JC, BJH, BL, WZ, YL, YZ, CW and XLY performed the experiments. AZ, MS, ARS, ZT, BJH, BL, WZ, YL, BY and ZLS analysed the data. ZLS and MS designed and supervised the overall study.

Compliance with Ethical Standards

Conflict of interests The authors declare that they have no conflict of interest.

Animal and Human Rights Statement This study was approved by the Wuhan Institute of Virology, Institutional Review Board (China) and by the Ethical Review Committee of Government College University Faisalabad (Pakistan). All institutional and national guidelines for the care and use of laboratory animals were followed and informed oral consent was obtained from humans for which identifying information is included in this article.

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