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# An observational, laboratory-based study of outbreaks of MERS-Coronavirus in Jeddah and Riyadh, Kingdom of Saudi Arabia, 2014

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**Summary**

In spring 2014, an explosive outbreak of MERS-Coronavirus in Jeddah caused conjectures about changes in viral transmissibility. Functional examination of circulating viruses as well as analyses of diagnostic laboratory data suggest causation by nosocomial transmission of a biologically unchanged virus.

**Abstract**

*Background*

In spring 2014, a sudden rise in the number of notified MERS-Coronavirus infections occurred across Saudi Arabia with a focus in Jeddah. Hypotheses to explain the outbreak pattern include increased surveillance, increased zoonotic transmission, nosocomial transmission, changes in viral transmissibility, as well as diagnostic laboratory artifacts.

*Methods*

Diagnostic results from Jeddah Regional Laboratory were analyzed. Viruses from the Jeddah outbreak and viruses occurring during the same time in Riyadh, Al-Kharj, and Madinah were fully or partially sequenced. A set of four single nucleotide polymorphisms distinctive to the Jeddah outbreak were determined from additional viruses. Viruses from Riyadh and Jeddah were isolated and studied in cell culture.

*Results and conclusions*

Up to 481 samples were received per day for RT-PCR testing. A laboratory proficiency assessment suggested positive and negative results to be reliable. Forty-nine percent of 168 positive-testing samples during the Jeddah outbreak stemmed from King Fahd Hospital. All viruses from Jeddah were monophyletic and similar, while viruses from Riyadh were paraphyletic and diverse. A hospital-associated transmission cluster, to which cases in Indiana/USA and the Netherlands belonged, was discovered in Riyadh. One Jeddah-type virus was found in Riyadh, with matching travel history to Jeddah. Virus isolates representing outbreaks in Jeddah and Riyadh were not different from MERS-CoV EMC/2012 in replication, escape of interferon response, and serum neutralization. Detection rates and average virus concentrations did not change significantly over the outbreak in Jeddah. These results suggest the outbreaks to have been caused by biologically unchanged viruses in connection with nosocomial transmission.

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## Introduction

The Middle East respiratory syndrome coronavirus (MERS-CoV) was discovered in 2012 and has since been found to cause sporadic cases and small case clusters of severe acute respiratory illness [1]. All patients occurred in the Arabian peninsula or had epidemiological links to the region. The total number of notified cases since 2012 was 199 as of 25 March 2014 [2]. From the end of March through April 2014 an exponential increase of new cases occurred in Saudi Arabia with a focus in Jeddah, causing conjectures about potential changes in fundamental epidemiological parameters [3]. Hypotheses to explain the outbreak pattern include increased surveillance, increased zoonotic transmission, increasing nosocomial transmission, changes in viral transmissibility, as well as false positive results due to laboratory errors. The latter option caused concern about the validity of the overall case count notified to WHO [3].

To fully appreciate the extensive outbreak in Jeddah, it will be necessary to reconstruct transmission chains and dissect the epidemiology in such a way that fundamental epidemiological parameters can be inferred. While these analyses may take considerable time, health authorities are in urgent need of information to guide potential alterations of preventive measures and travel recommendations. Virological studies can provide valuable insight into virulence and transmissibility even in absence of detailed clinical or epidemiological information. Moreover, the trend in numbers and nature of requests received in the diagnostic laboratory can provide helpful insight into the general situation at point of care.

During the outbreak in Jeddah, all RT-PCR testing was centrally performed by Jeddah Regional Laboratory (JRL). JRL is a reference facility within the laboratory network of the Saudi Ministry of Health that serves the Jeddah region and provides confirmatory MERS-CoV testing for all Ministry of Health laboratories across the Kingdom. Here we provide direct insight into laboratory results from JRL and performed a thorough analysis of the outbreak-associated virus along with functional studies of virulence and immune escape in cell culture. We compare Jeddah-derived viruses with viruses occurring elsewhere in the country during the same time.

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## Materials and Methods

*RT-PCR and sequencing*

All procedures followed protocols described previously [5-7]. JRL used LightMix kits (TIB Molbiol) containing pre-mixed primers and probe for the upE and ORF1A assays to minimize the risk of reagent-based contamination and detection artifacts [5]. Primers for viral genome sequencing are available upon request.

*Virus isolation*

Samples were inoculated in VeroB4 cells seeded at $3x10^5$ cells/mL in 24well plates 16h prior to infection, for 1 h at 37°C. Cells were incubated at 37°C and checked daily for cytopathogenic effects. Every 2 days, cell culture supernatant was sampled and tested by real-time RT-PCR for increase of MERS-CoV-specific viral RNA. PCR positive wells were harvested and used for the production of virus stocks. Virus stocks were quantified by plaque titration on VeroB4 cells as described earlier [8].

*Virus growth kinetics*

A549 cells (ATCC CCL-185) were seeded at $2x10^5$ cells/well in 24 well plates 16 h prior to infection. At 1, 8, 24, 48 and 72 h post infection, supernatants were sampled and the increase of MERS-CoV-specific viral RNA quantified by real-time RT-PCR [8].

*Plaque titration and neutralization assay*

VeroB4 cells were seeded at $1.5x10^5$ cells/well in 24 well plates 16 h prior to titration. Cells were overlaid after infection with 500 µL Avicel (FCM BioPolymer) at a final concentration of 1.2% in DMEM [9]. Three days post infection, cells were fixed in 6% formaldehyde and stained with crystal violet solution. For neutralization assay [10, 11], 25 plaque forming units of MERS-CoV were pre-incubated with diluted serum for one hour at 37°C .

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## Results

*Laboratory performance and overall diagnostic results*

Case identification and notification during the outbreak in Jeddah was mainly based on laboratory testing. To obtain insight into laboratory testing during the outbreak, the sample reception list in JRL was analyzed (**Figure 1**). There was a striking increase of diagnostic requests during April which was mainly caused by samples from Jeddah (**Figure 1A**). From January 1st to April 28th, JRL received 6,285 samples for RT-PCR testing for MERS-CoV. 5,828 of these samples were received only since March 26th, the date when the first case in the Jeddah outbreak was tested. This suggests a 36.8-fold increase of the monthly workload in April. The maximal number of samples received in a single day was 481. Almost half of all positive testing samples during the Jeddah outbreak (82 of 168) stemmed from King Fahd Hospital. The rate of samples with positive tests from King Fahd Hospital seemed to increase earlier than in other hospitals in the city (**Figure 1B**). Over the course of four weeks in April, the fraction of positive RT-PCR results in samples from Jeddah as well as samples from all cities did not vary significantly (**Supplementary Table 1**). While the laboratory entry list did not identify the symptoms status of patients, it indicated by presence of a patient identifier code whether cases were in hospital or likely part of a contact investigation (**Table 1**). There was a marked increase of contact investigations in Jeddah versus other locations. Expectedly, the proportion of samples with low viral loads (indicated by high Ct values) was high in contact investigations (**Figures 1C and D**).

Studies of reliability of laboratory procedures as presented in **Supplementary dataset 1** did not reveal any evidence for generic background contamination in the laboratory.

*Viral genome sequence and phylogeny*

Seven viruses from the Jeddah outbreak were entirely sequenced and compared with full-length or subtotal genome sequences available in April 2014 in GenBank (**Supplementary Table 2**). An analysis of major reading frames across the genome taking into account additional spike gene sequences (KM027263-KM027276) suggested no unique amino acid changes in relevant protein domains (**Supplementary Dataset 2**).

All viruses pertaining to the Jeddah outbreak clustered in one phylogenetic clade (**Figure 2**). Seventeen partial genome sequences were determined from samples

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obtained from Riyadh, Al-Kharj, and Madinah during March and April 2014 for comparison. These partial sequences comprised the entire structural protein genes of the MERS-CoV genomes, ca. 8.7 kB in length. As shown in **Figure 2**, viruses from Riyadh fell into 6 different positions, one of which (clade 2) may constitute a human-to-human transmission cluster to which also the exported cases to Indiana/USA as well as the Netherlands belong (**Supplementary Table 3**)[12, 13]. Another virus from Riyadh clustered with Jeddah-type viruses. This patient originated from Jeddah and had visited his sick son in King Fahd Hospital in Jeddah before his trip to Riyadh.

To better evaluate the diversity of viruses circulating in Jeddah, single nucleotide polymorphisms (SNP) were studied (**Table 2**). All samples except one had the same combination of SNPs. The one deviating sample was taken on April 22nd and had a double peak in one SNP that was confirmed twice by repetition of RT-PCR and sequencing. Further partial sequencing of this virus did not yield any other double peaks, suggesting the ongoing formation of quasispecies as described before [14], rather than simultaneous infection with two viruses. The sequences from a US case and a case in Riyadh with known travel histories to Jeddah had Jeddah-typical SNP patterns (**Table 2**). In contrast, viruses detected in Jeddah one month and 5 months before the outbreak did not cluster with the Jeddah-type outbreak viruses. A virus detected in Riyadh (SA2014_158) was related to camel viruses sharing a recent common ancestor with Jeddah-type outbreak viruses, but was distinct in its SNP pattern.

*Virus infection studies*

To study potential alterations in virus functions, 16 clinical samples from Jeddah with projected viral loads of $5x10^6$ copies per sample or higher were selected and inoculated in Vero B4 cells. Five viral isolates were obtained. Because the replication phenotype of all viruses was highly similar in preliminary experiments, one isolate termed MERS-CoV Jeddah_10306 was fully sequenced and chosen for further study (GenBank No KM027260, **Supplementary Table 2**). For comparison, virus was isolated from patients in a hospital-associated cluster in Riyadh and an isolate termed MERS-CoV Riyadh_683 was chosen and sequenced (GenBank No KM027262, **Supplementary Table 3**). The original viral isolate EMC/2012 [1] was compared as well.

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Single step growth curves were done on Vero cells by inoculation with high multiplicities of infection (MOI) of 1 infectious dose per cell, which will reveal gross differences such as in the viruses´ capacity to enter cells. As shown in **Figure 3A**, there were no relevant differences in replication between the three viral strains. Because Vero cells derived from rhesus monkey kidney tissue might not optimally reflect the target tissue of MERS-CoV infection, A549 cells derived from a human alveolar epithelial carcinoma (non small cell lung cancer) were used in parallel. Results of one-step growth curves were highly similar (**Figure 3B**).

Because differences in the viruses´ adaptation to replicate in primate cells may not become obvious in one-step growth curves, replication trials were repeated in parallel in both cell lines using a reduced MOI of 0.01 that causes a prolonged course of replication with multiple rounds of infection in culture. No relevant difference in replication was seen between all 3 viral isolates in Vero and A549 cells (**Figure 3C and D**).

The type I interferon system is among the most efficient innate antiviral defenses. As MERS-CoV EMC/2012 was shown to be highly susceptible against type I interferon, infection trials were done in Vero cells pre-treated with interferon alpha to induce an antiviral state prior to infection in cells at MOI = 0.01. Even though Vero cells are known to induce an efficient antiviral state upon external IFN stimulus, no differences between the three viral strains were seen (**Figure 3E**).

Antibody functions provide a laboratory correlate of adaptive immunity. As viruses may differ in their robustness against neutralizing antibodies, all three viruses were subjected to plaque reduction neutralization assays using serum of a MERS patient with known antibody titer [7]. No relevant differences in the reduction of viral plaques depending on serum dilution were seen with any virus (**Figure 3F**).

*Viral loads*

Viral load data reflect clinical virus excretion, which cannot be modeled in cell culture. Ct values as a surrogate of viral loads were compared between samples from Jeddah and other cities (**Figure 4 A and B**). Mean Ct values in Jeddah and elsewhere were not significantly different (30.4 and 31.4, respectively). However, the frequency

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distributions and median values suggested a pronunciation of lower viral load samples in Jeddah. Within Jeddah, Ct values in KFH (n=82) were not different from those in any other hospitals (n=108). All samples from Jeddah tested during April were categorized by week of reception and plotted as shown in **Figure 4C**. There was a subjective trend toward lower Ct values by the third week. However, these points were identified as outlier values and mean viral loads did not differ significantly in any of the weeks of April according to ANOVA analysis ($F=0.82$, $p=0.48$). One of those outlier samples with a very low Ct value encountered on April 20th, 2014 yielded the isolate of MERS-CoV C10306, which has been entirely sequenced without any evidence for significant mutations, and which was studied in above-described cell culture experiments without any evidence for increased virulence.

**Discussion**

The unprecedented increase in new cases of MERS-CoV infections during spring 2014 has caused concern in the public health community worldwide. Our initial sequence analyses communicated during the ongoing outbreak provided a preliminary idea of the molecular epidemiology with outbreak viruses forming a homogeneous, monophyletic clade [4]. Paraphyly of concurrent viruses is expected when infections are independently acquired from a diversified source population such as expected in animal reservoirs. In Riyadh, concurrently circulating viruses were indeed distributed across at least six different clades, suggesting these infections to result from increased zoonotic activity or introduction of human viruses from other regions. One larger virus cluster was observed in Riyadh, associated with one specific hospital suggesting nosocomial transmission (clade 2). The case exported to Indiana/USA had worked in this hospital while the cases in the Netherlands were hospitalized in Madinah but not Riyadh[12, 13]. This suggests unnoticed transmission links such as infected patients transferred between hospitals, or acquisition from common zoonotic sources.

Interestingly, one of the viruses seen in Riyadh resembled camel viruses in close relationship to Jeddah-type strains. These viruses may have been widely distributed in camels by late 2013 to early 2014, as they were detected in Taif southwest of Jeddah and in Qatar on the eastern Arabian Peninsula [14, 15]. Viruses encountered in Jeddah shortly before the outbreak such as Jeddah-1 or Jeddah_C6664 were clearly distinct, suggesting that the outbreak might have been initiated by the introduction of Jeddah-

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type viruses into camels in the region. The monophyly and similarity of outbreak viruses favors the idea that the subsequent transmission took place in humans. The regional restriction of outbreak viruses matches our earlier observation of low transmissibility between humans in non-nosocomial settings such as household contact clusters [16]. In spite of a documented transmission from Jeddah to the capital Riyadh, there was no evidence of further human-to-human spread in Riyadh. From the analysis of SNP patterns it was concluded that all Jeddah-type viruses were homogenous without evidence for concomitant circulation of other strains during the outbreak. Nevertheless, our preliminary sequencing studies found no relevant genetic changes sufficient to explain an altered epidemic pattern [4]. As we have now been able to isolate live viruses, we can provide a first side-by-side comparison of different viral strains of MERS-CoV. Of note, these virus isolates were representative of two likely nosocomial outbreaks in Jeddah and Riyadh, both causing international spread of the virus to the USA, the Netherlands, as well as Greece. Cell culture experiments yielded no evidence for changes in viral replication or immune escape. The absence of differences in serum neutralization disfavors antigenic variability as a promoter of transmissibility. As the selected viruses represent major branches of the known MERS-CoV tree, these data additionally suggest the absence of serotypes in MERS-CoV, which is reassuring regarding the prospects to develop immunization approaches.

By the end of the outbreak late in April 2014, the accumulation of laboratory data at JRL allowed first insights into shedding properties of circulating virus, which compensates for the inability of cell culture to reflect virus transmissibility. We have obtained no evidence suggesting that concentrations of shed virus might have changed. A subjective trend toward higher peak (but not average) concentrations later into the outbreak may be explained by increased disease awareness in hospitals leading to an earlier investigation of suspected cases. Similar observations were made during the SARS epidemic in Hong Kong where cases were detected earlier after some time into the outbreak [17]. The absence of changes in average virus concentrations makes it unlikely for the virus to have changed its transmissibility and virulence over the course of the outbreak.

The reason for the explosive nature of the epidemic in Jeddah may thus be found elsewhere, such as in the rate of human-to-human contact. In this light, our analysis of

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laboratory statistics is highly suggestive of an outbreak fuelled by the healthcare setting. Not only did about half of all patients with a positive diagnosis pertain to one particular hospital, but also the first peak case counts in this hospital predated increases elsewhere, and new peaks were followed by peaks of cases in other hospitals. This pattern is highly suggestive of an epidemiological hotspot where the virus is amplified and from where limited transmission chains are seeded. Indeed, King Fahd Hospital is the largest communal hospital in Jeddah serving as the primary care center for all patients attending the MOH healthcare system, as well as for a large fraction of expatriate workers in the city. It is reassuring that the number of new cases in King Fahd Hospital came down toward the end of the study period. This trend started even before changes such as the closure of emergency rooms and the transfer of infected patients were implemented, pointing to the possibility that transmission may have been limited mainly by heightened awareness of the disease among health care workers and patients. Again, a similar effect has been documented during the SARS epidemic in Hong Kong [17].

An important observation in case notifications during the outbreak was the increase of cases notified as "asymptomatic" or "mild" in the Jeddah case statistics. As shown in our assessment of sample receptions, the huge amount of laboratory requests during peak phases of the epidemic caused an overload on laboratory capacities without a significant increase of the fraction of requests that were confirmed virus-positive. A low predictive value of clinical suspicion is caused by an insufficient case definition or lack of adherence to the case definition, such as suggested by a high fraction of tests in cases without proper hospital registration number. Unjustified RT-PCR testing raises the likelihood of human error. As far as possible, we have assessed the technical capabilities of JRL and found no general issues of cross-contamination. Nevertheless, we cannot exclude issues elsewhere in the logistics chain, such as near the bedside where diagnostic samples may have been handled in bulk. The high similarity of all Jeddah-type viruses will make it impossible to resolve potential contamination sources retrospectively by sequencing of stored samples. Nevertheless, a certain rate of positive test results in asymptomatic persons might be considered plausible as unnoticed replication has been shown for SARS-CoV whose RNA was detected in exposed healthcare workers with no or mild symptoms, as well as in our recent study on

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household contacts of MERS-CoV cases [16, 18]. Such replication may be transient, and the low viral loads seen in contacts might not suffice to establish infection chains.

In conclusion, our investigations suggest a predominance of human-to-human transmission during the Jeddah outbreak without evidence for modification of viral shedding, replication, and immune escape. A coincident increase of cases in Riyadh was the result of multiple, independent, sources with some phylogenetic evidence of nosocomial spread. Contact tracing by RT-PCR should be restricted to defined groups of patients to avoid an overload on the health care system. Retrospective serological tests may provide a valid alternative to RT-PCR testing of contacts [16].

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**CONFLICTS OF INTEREST**: Olfert Landt is CEO of TibMolbiol, a company providing some of the RT-PCR reagents used in this study. He or his company had no influence in the decision to use these reagents. The work does not make any comparisons of these reagents with products provided by competing commercial or noncommercial entities. All other authors declare no conflicts of interest.

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**Table 1. Tests in samples with and without hospital number, by City**

| City | Tests with hospital number | Tests without hospital number[a] | Ratio |
|---|---|---|---|
| Jeddah | 3739 (4% positive) | 1056 (1.7% positives) | 28% |
| Non-Jeddah | 1072 (2.9% positive) | 59 (0 positives) | 5.5% |

a: These cases were enlisted with no hospital number but carried either of the following identifiers: "Contact", "HCW", or had a cell phone number entered in the identifier field that the laboratory was asked to call in case of self-initiated diagnostic tests by physicians or their family members (n=41).

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**Table 2. Single nucleotide polymorphisms in Jeddah-type viruses and reference viruses**

| Sample ID | Sample/patient origin | Sampling date | SNP position in EMC/2012 genome | | | |
|---|---|---|---|---|---|---|
| | | | 737 | 17836 | 23953 | 28778 |
| 68 samples from JRL[a] | Jeddah, Makkah | 26 Mar to 23 Apr 2014 | C | T | G | A |
| Human\|2014SA_693[b] | Riyadh | 22 Apr 2014 | C | T | G | A |
| Human\|Florida/USA-2/Jeddah | Jeddah | 10 May 2014 | C | T | G | A |
| Human\|C10829 | Jeddah | 22 Apr 2014 | C | T | G | A/T |
| Camel\|Qatar_2\|KJ650098 | Qatar | 16 Feb 2014 | C | C | A | T |
| Human\|C6664[c] | Jeddah | 18 Feb 2014 | T | C | ? | T |
| Human\| 2014SA_158[d] | Riyadh | 20 Mar 2014 | T | C | A | T |
| Camel\|Jeddah_1_2013\|KJ556336[e] | Jeddah | 6 Nov 2013 | T | C | A | T |
| Camel\|KSA-505\|KJ713295 | Taif | Nov 2013 | T | C | A | T |
| Camel\|KSA_378\|KJ713296 | Taif | Nov 2013 | T | C | A | T |
| Human\|2014SA_683 | Riyadh | 21 Apr 2014 | T | C | A | T |
| Camel\|KSA-503\|KJ713297 | Taif | Nov 2013 | T | T | A | T |
| Camel\|KSA-363\|KJ713298 | Taif | Nov 2013 | T | T | A | T |
| Human\|EMC/2012\|JX869059 | Bisha | Jun 2012 | T | C | A | T |

a: Median sampling date on 14. April. The 68 samples represented 40% of all positive samples identified at JRL in Jeddah patients

b: This patient had a travel history to King Fahd Hospital in Jeddah within one incubation time before onset of symptoms

c: This was the last patient detected and sequenced in Jeddah before the onset of the outbreak end of March. The SNP at position 23953 could not be sequenced because the diagnostic sample contained only minute amounts of RNA and had been stored at -20°C for prolonged time.

d: This patient had no travel history. Virus 2014_SA158 clusters amongst camel viruses in ancestral relationship to Jeddah-type human viruses, such as Camel_Qatar2_KJ650098.

e: This virus was transmitted from a camel in Jeddah, October/November 2013

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**Legends to Figures**

**Figure 1. Summary of features of the outbreak as derived from JRL lab file data.** A, overall diagnostic requests; B, positive cases (y scale = cases per day) in King Fahd Hospital versus all other hospitals, recording 3-day intervals starting on March 26 and ending on April 28. C and D: Distribution of Ct values in 1056 samples pertaining to investigations in cases without hospital number in Jeddah (n=18 positive samples), versus 3799 samples with hospital number (n=150 positive samples). Average Ct values in cases and contacts were 30 and 33.1, respectively (2-tailed t-test, $p<0.009$).

**Figure 2.** Phylogenetic tree inferred using MrBayes [20] for the concatenated coding regions of 105 MERS-CoV genomes or partial genomes sampled from humans and camels. We employed a codon-position-specific GTR substitution model with gamma-distributed rates amongst sites. Displayed is the majority-consensus of 10,000 trees sampled from the posterior distribution with mean branch lengths. Posterior support is shown for nodes where less than 0.90. Sequences sampled from camels are denoted with a yellow circle, those from humans with a green circle. Sequences new to this study are labelled in bold. The cluster comprising viruses isolated from the Jeddah/Makkah hospitals in April 2014 are highlighted with a red box and those from the Prince Sultan Military Medical City, Riyadh in March, April 2014 are highlighted in blue. For comparison the Al-Hasa 2013 hospital outbreak [21] is highlighted in yellow and the 2013 Hafr-Al-Batin community outbreak [22] in green.

**Figure 3: Growth kinetics of MERS-CoV** EMC/2012, Jeddah_10306, and Riyadh_683 in cell culture. VeroB4 and A459 cells were infected at MOI 1 (A and B, respectively) or MOI 0.01 (C and D, respectively). Samples from the supernatant were taken at indicated time points and virus growth was measured by real-time RT-CPR. VeroB4 cells infected at MOI 1 (A) showed total cytopathogenic effect 48 h post infection, terminating the experiment. A459 cells did not show any CPE even when infected at MOI 1 at 72 h p.i. (B). E, effect of pretreatment of cell cultures with type I interferon at low or high dosage. D, virus neutralizing effect of human serum with known anti-MERS-CoV neutralizing antibody titer at different dilutions.

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**Figure 4.** Virus shedding in patients. Ct values during the outbreak in Jeddah. A and B, frequency distribution of Ct values in Jeddah versus other cities; C, Ct values during the outbreak in Jeddah by week, starting on March 26th, 2014.

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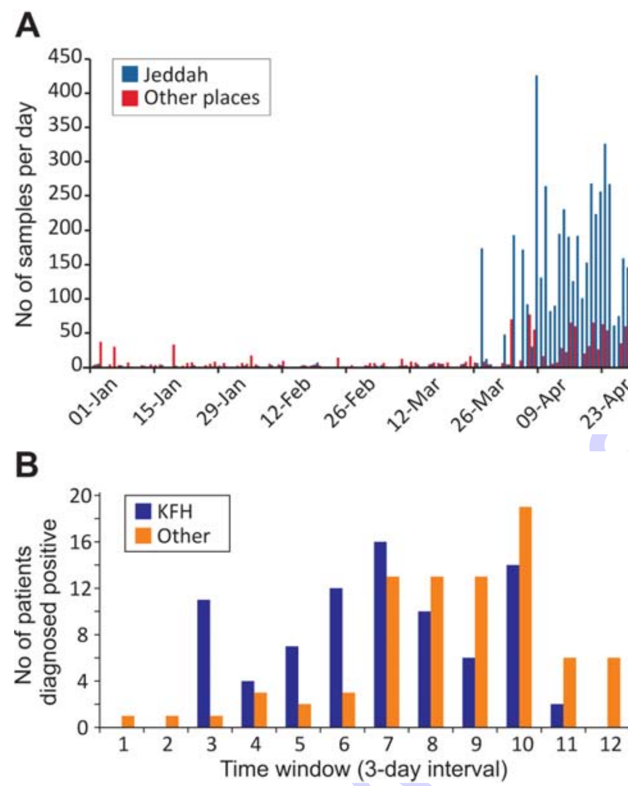


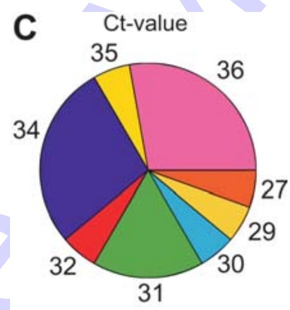


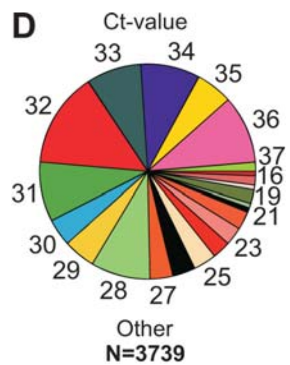


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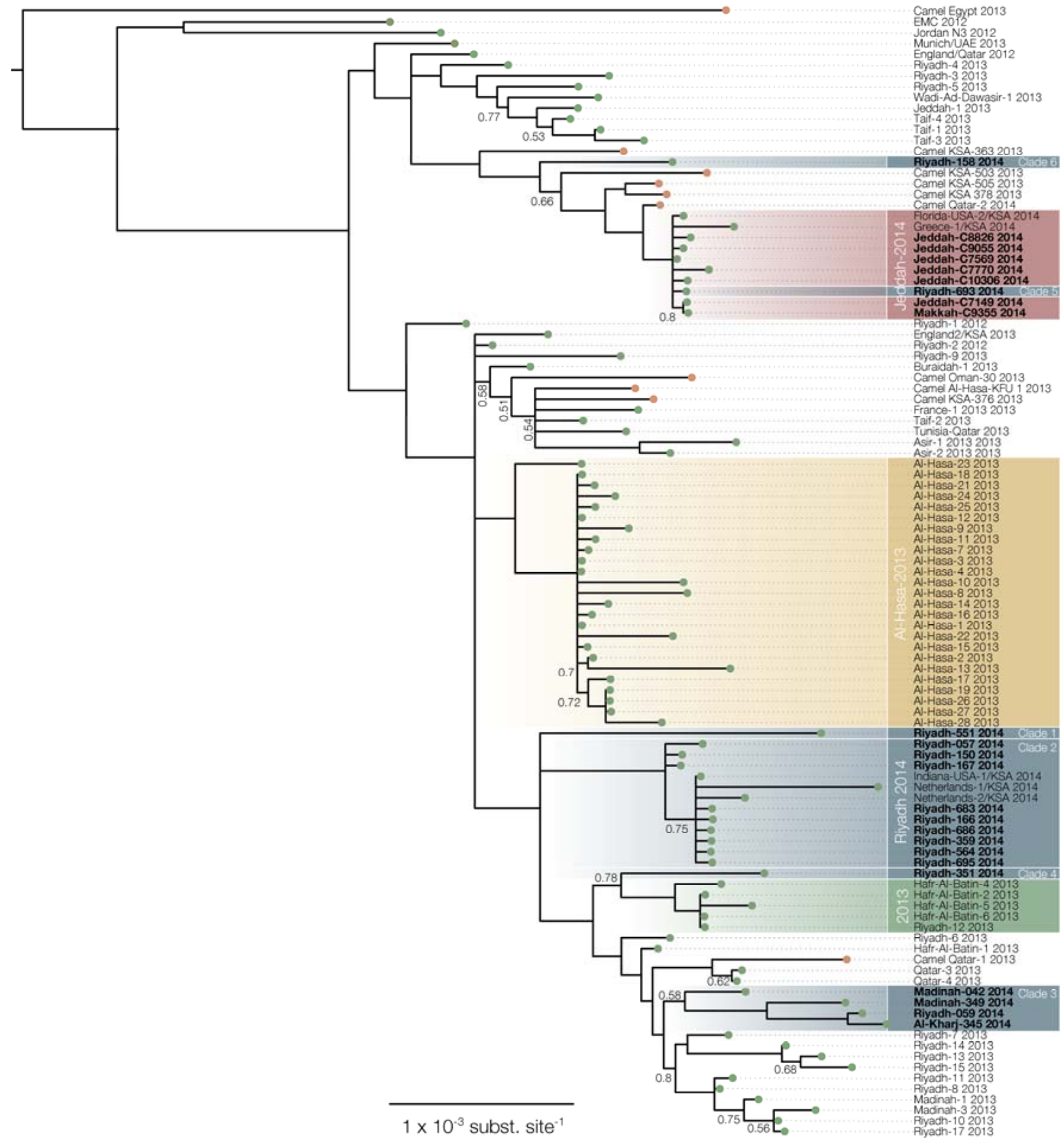


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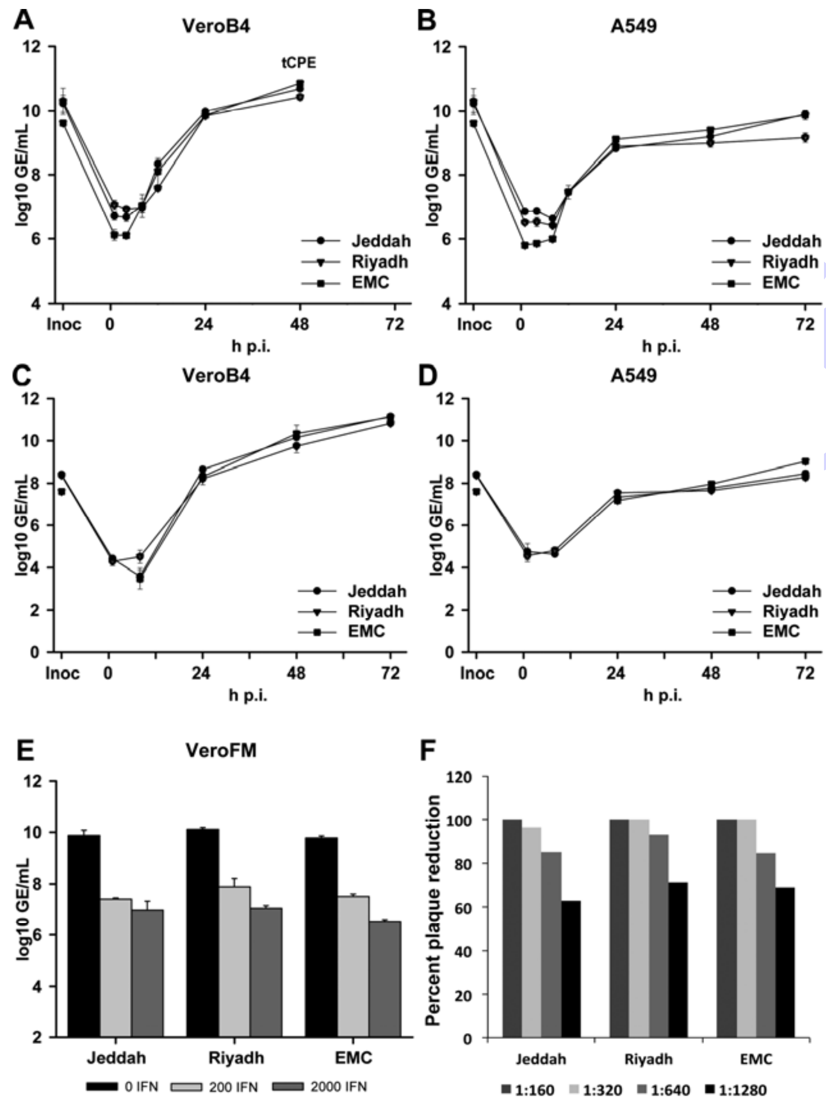


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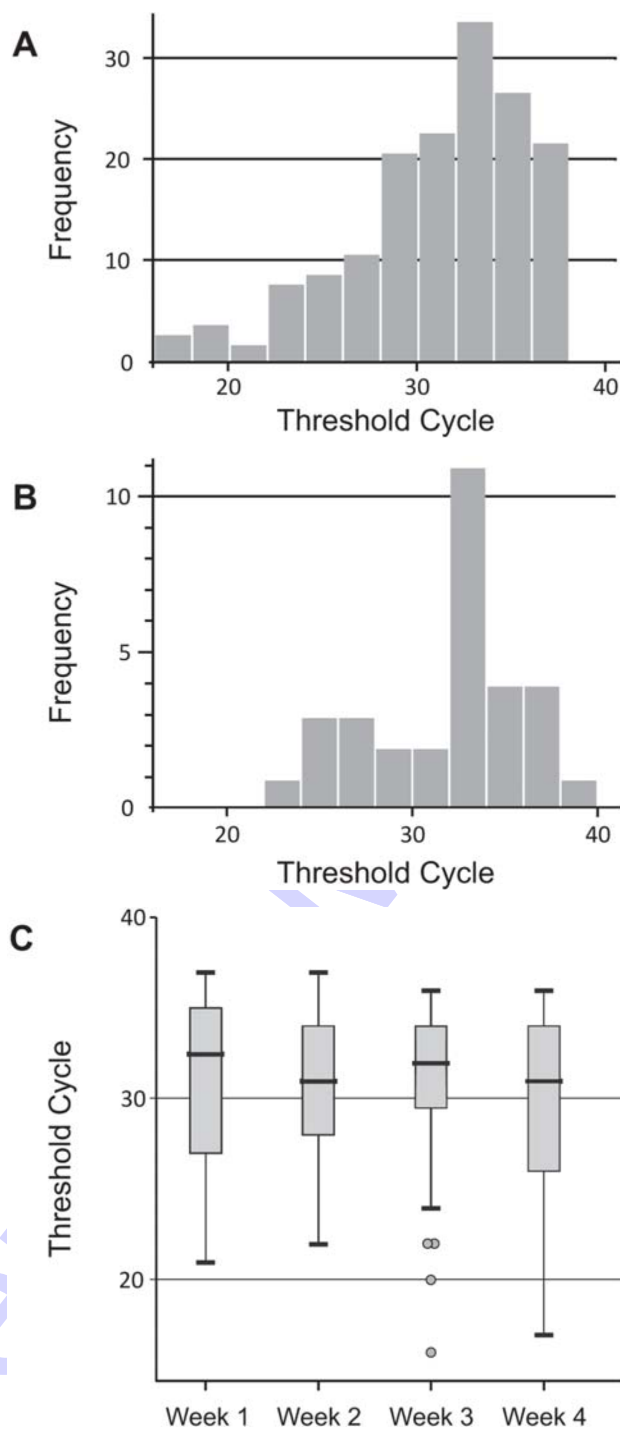


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