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Rapid one-step construction of a Middle East Respiratory Syndrome (MERS-CoV) infectious clone system by homologous recombination

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Background: Viral infectious clone systems serve as robust platforms to study viral gene or replicative function by reverse genetics, formulate vaccines and adapt a wild type-virus to an animal host. Since the development of the first viral infectious clone system for the poliovirus, novel strategies of viral genome construction have allowed for the assembly of viral genomes across the identified viral families. However, the molecular profiles of some viruses make their genome more difficult to construct than others. Two factors that affect the difficulty of infectious clone construction are genome length and genome complexity.

Results: This work examines the available strategies for overcoming the obstacles of assembling the long and complex RNA genomes of coronaviruses and reports one-step construction of an infectious clone system for the Middle East Respiratory Syndrome coronavirus (MERS-CoV) by homologous recombination in S. cerevisiae.

Conclusions: Future use of this methodology will shorten the time between emergence of a novel viral pathogen and construction of an infectious clone system. Completion of a viral infectious clone system facilitates further study of a virus’s biology, improvement of diagnostic tests, vaccine production and the screening of antiviral compounds.

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1. Background

In June of 2012, the Middle East Respiratory Syndrome (MERS) coronavirus (CoV) was isolated from a patient suffering from symptoms of respiratory disease in Jeddah, Kingdom of Saudi Arabia (Zaki et al., 2012). MERS-CoV genetically clusters with the bat coronaviruses Neoromicia/PML, HKU4-1 and HKU5-1 which occupy lineage C of the betacoronaviridae, no other identified human coronavirus exists within this genus (Raj et al., 2014). Epidemiological studies of MERS-CoV implicate camels as a possible zoonotic host (Memish et al., 2014; Reusken et al., 2013). Since its emergence, MERS-CoV has spread between 26 countries causing 1375 laboratory confirmed cases and 587 deaths (ECDC, 2015; ProMED-mail, 2016). No other coronavirus has so greatly impacted public health, or global economics since severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 (Hilgenfeld and Peiris, 2013). The unexpected introduction of SARS-CoV and MERS-CoV into the human population combined with a lack of current antiviral therapies or vaccines to protect the public from coronavirus related disease, makes study of these and similar viruses important to public health. In order to improve the timeline between discovery of a novel coronavirus and completion of an infectious clone system, we undertook the rapid construction of the MERS-CoV cDNA genome EMC/2012 (JX869059.2) by homologous recombination in S. cerevisiae.

Virus infectious clone systems allow for the expression of a homogenous virus population within mammalian cell culture from a sequence of DNA or RNA (Ebihara et al., 2005). The construction of such systems for RNA viruses involves several steps: 1) pos...

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session of a virus isolate, 2) sequencing of the viral genome, 3) reverse transcription of the viral RNA genome to complementary DNA (cDNA) and 4) assembly of the PCR cDNA amplicons into the full-length genome. The timing and technical expertise required to perform these steps depends on the type of viral genome being constructed. Infectious clone systems for viruses with small unfragmented positive sense RNA genomes are less demanding to construct than those with large negative sense, positive sense or fragmented genomes (Ebihara et al., 2005; Yount et al., 2000; Stobart and Moore, 2014).

Construction of infectious clone systems for viruses of the Coronaviridae has lagged in comparison to those of other viral families because qualities of the coronavirus genome restrict the use of traditional molecular cloning techniques (Stobart and Moore, 2014). The prototypical coronavirus possesses a positive sense RNA genome from 27 to 32 kb in length. Multiple regions of sequence redundancy exist (Gorbalenya et al., 2006), which decrease the availability of unique restriction sites. The MERS-CoV genome (JX869059.2) has only 12 unique restriction sites (Fig. 1a); their positions and the sizes of the corresponding DNA fragments preclude a simple cloning strategy. Highly cytotoxic regions have also been described within coronavirus genomes, which cause E. coli cloning strains to poorly tolerate plasmids carrying coronavirus genome cassettes (González et al., 2002; Yount et al., 2002). Performing polymerase chain reaction (PCR), especially reverse transcription PCR (RT-PCR) on coronavirus genomes proves troublesome, as the error rate of DNA polymerases increases in relation to copy number and amplicon size (Eckert and Kunkel, 1991).

In combination, these factors make the assembly of coronavirus genome’s by traditional molecular cloning tedious and time consuming (Almazán et al., 2014; Enjuanes, 2005). Virologists have developed several alternative methods to overcome these obstacles; however, none of them allow for rapid construction and expression of a coronavirus infectious clone system. The attempted strategies include targeted RNA recombination, transfection of multiple or single plasmid vectors and vaccinia virus transduction. Almazán et al. conducted a concise summary of available strategies for coronavirus genome construction in their 2014 review. In summary, they concluded that several alternatives to conventional approaches have benefited the science of coronavirus genome construction over the last thirteen years. We hypothesize that construction of a coronavirus genome by homologous recombination within S. cerevisiae, will prove an additional efficient means to molecular cloning coronaviruses while preserving the strengths of the plasmid base method: long term stability and efficient expression (Fig 1c) (Almazán et al., 2014).

2. Methods

2.1. Chemical synthesis of MERS-CoV cDNA, plasmid selection and primer design

Thirty-one, 1-kb gene fragments of the MERS-CoV EMC/2012 genome (NC_019843.2, JX869059.2) were chemically synthesized and cloned into the EcoRI sites of pCR® 2.1 (Life Technologies) by Eurofins MWG Operon. The pYES1L-BAC vector (Life Technologies) was chosen for conducting homologous recombination in S. cerevisiae and maintenance of the completed MERS-CoV infectious clone system in E. coli. Primers were designed for stepwise assembly of the synthetic MERS-CoV gene fragments and amplifying products for homologous assembly in S. cerevisiae (Table 1.0 and Supplementary Table 1.0).

2.2. Assembly of MERS-CoV cDNA by polymerase chain reaction

The 1-kb MERS-CoV gene fragments and regulatory genetic elements (i.e. cytomegalovirus promoter, beta globulin terminator and hepatitis delta virus ribozyme) were stepwise assembled
by splice-by-overlap extension polymerase chain reaction. Three large PCR amplicons were produced by this method ranging from: (MF1) CMVpF Start (TATTA)- EMC/2012 (6023 bp), (MF2) EMC/2012 (5981 bp)-EMC/2012 (18,020 bp) and (MF3) EMC/2012 (17,978 bp)- Beta-Globulin Terminator end (TGCTC). The three templates MF1, MF2 and MF3 were PCR amplified using primers with ~30 bps of homology to the neighbouring fragment or blunt ends of the pYES1L vector (Fig. 1b) (Table 1) with PrimeSTAR® GXL polymerase. The resulting amplicons were purified by gel electrophoresis and extracted using the NucleoSpin® kit (Macherey-Nagel). The elution buffer NE was pre-warmed to 70 °C to increase the yield of purified DNA from the silica membrane spin columns. MF1, MF2 and MF3 were not subcloned into maintenance vectors, instead they were transfected directly into the yeast cells for homologous recombination.

### 2.3. Homologous recombination of MERS-CoV cDNA

Homologous recombination of the three MERS-CoV gene fragments (MF1, MF2 and MF3) and the pYES1L-BAC vector was performed using the GeneArt® High-Order Genetic Assembly System (Life Technologies). Chemically induced MaV203 cells (Life Technologies) were transformed with 100 µg of pYES1L Vector, 173 ng of MF1, 205 ng of MF2 and 100 ng of MF3. Transformed cells were grown on complete supplement mixture deficient in tryptophan (CSM-Trp) agar plates for three days at 30 °C. The resulting colonies were replica plated and screened for presence of the MERS-CoV genome by PCR with the GoTaq® polymerase (Promega). S. cerevisiae cells were disrupted by suspension in 30 µL of 0.2% w/v sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and heat treatment at 95 °C for 5 min. The samples were diluted with 20 µL of

#### Table 1

Oligo-nucleotide primers used for assembly of MERS-CoV cDNA by homologous recombination.

| Primer Name | Use | Sequence (5’-3’) |
|-------------|-----|------------------|
| CMVpF       | MF1-F | CGCTGATACGGCCGCTATTTAAATAGTAACTATAGCGTACGCTTAT |
| M-CoV6, 000R| MF1-R | ATCTCTTATAATAGGCTTAAGTCTGCAACTCACCACAAAGGCAAA |
| M-CoV6, 001F| MF2-F | GTGCGCTGAGTTGACATCTAGCTGACCCATTATCTAAAGAAGG |
| M-CoV18, 000R| MF2-R | GGCGAATTAAATCTGACAGCTGACGGTCTTCTAATAGGAAAGA |
| M-CoV18, 001F| MF3-F | TCTTTTCTAATTACAAGCGCTCCAGCTCAGTTGATGTCGG |
| BGTR        | MF3-R | TAACTGGGCGGAGCCGAGCATGCTGATGTCCTCCAAAATCACACCAATAC |
| pYES1L F    | Vector | CATATGCAATATGTCGCCGAGCATATTCAAAGGTTTC |
| pYES1L R    | Vector | TGATCTATTACATAGCGGCGCTATCAGG |

Primers containing ~30 bps of homology between MERS-CoV cDNA fragments (MF1, MF2 and MF3) and the pYES1L-BAC vector were used to amplify the regions in preparation for assembly by homologous recombination.

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**Fig. 2.** (a, b) Transformed S. cerevisiae colonies were screened for the MERS-CoV/pYES1L plasmid vector by PCR (Primer Set 1: F- GTGCTGATTGAGACTCATCATCACATCAATTAAAGAACATCATATA and R- AGTGGGAGACCTATCCGCATATTTACAAAGGAC, Primer Set 2: F- AGTGGGAGACCTATCCGCATATTTACAAAGGAC and R-TGTGAGGCTGATGAGCTGATGAG). (c) Cellular supernatant was withdrawn from cells infected with either rescued rMERS-CoV virus or rescued rSARS-CoV virus and used in a RT-PCR assay to detect a region of the MERS-CoV polymerase gene (Primer set: F- CAAGATGAACTTGGCCACAAAGGTAAAGGCTCATTCA and R- TGTGAGGCTGATGAGCTGATGAG). The MERS-CoV polymerase gene was detected in Lane 1 (rMERS-CoV supernatant) and not Lane 2 (rSARS-CoV supernatant). Lane 3 serves as a positive control, it shows PCR amplification of the MERS-CoV polymerase gene from the infectious clone system. (d) The polymerase gene fragment amplified from rMERS-CoV infected cell supernatant was sequenced and used in a phylogenetic comparison (ClustalW Algorithm) with six coronaviruses: CoV-HKU4-1 (NC_0009019.1), CoV-HKU5-1 (NC_0009020.1), SARS-CoV/Tor 2 (NC_004718.3), CoV-Neoromisia/PML (KC_859678.4), CoV-229E (NC_002645.1) and MERS-CoV/EMC/2012 (NC_019843.3).
nuclease-free water and chilled on wet ice. Half of a microliter of lyzed S. cerevisiae colony supernatant was used as a template in PCR reactions. Positively screened colonies were grown in 5 mL volumes of CSM-Trp liquid media and the pYES1L-BAC/MERS-CoV plasmids were isolated using the Easy Yeast plasmid Isolation Kit (Takara, Clontech). A 5 μL volume of isolated plasmid was electroporated into Max Efficiency DH10B cells (Life Technologies). DH10B colonies were screened by PCR with GoTaq® polymerase and positive pYES1L-BAC/MERS-CoV plasmids were isolated using the HiSpeed Plasmid Maxi Kit (Qiagen). To view the primers used for the screening reactions refer to the caption of Fig. 2 (Fig. 2a and b).

2.4. Sequencing of the MERS-CoV genome

The assembled MERS-CoV genome was sequenced by Sanger sequencing at the DNA Core Facility, Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba. The sequences were aligned and interpreted using the SeqMan Pro software of the DNASTAR Lasergene 11 Core Suite.

2.5. Rescue of infectious MERS-CoV virus from the infectious clone system

Functionality of the MERS-CoV infectious clone system was examined by rescue in mammalian cell culture. Baby Hamster Kidney cells (BHk-21) (ATCC: CCL-10™) were grown in a T75 flask (NUNC) in Eagle’s Minimal Essential Medium (EMEM) supplemented with 5% v/v fetal bovine serum and 1% v/v penicillin/streptomycin (HyClone) to full confluence at 37°C 5% CO₂. Transfection complexes of pYES1L-BAC/MERS-CoV plasmid (300 ng) were prepared in Opti-MEM™(Life Technologies) using Fugene® (Promega) transfection reagent and added to a 6-well plate of 70% confluent BHK-21 following 15 min of incubation at room temperature. The transfected BHK-21 cells were incubated overnight at 37°C 5% CO₂. They were then treated with 0.25% v/v trypsin-EDTA(Gibco™) and a suspension of BHK-21 cells was overlaid onto a monolayer of Vero Cells (ATCC: CCL-81) grown to ~80% confluence in a T75 flask with Dulbecco’s modified eagle medium (DMEM) supplemented with 5% v/v fetal bovine serum and 1% v/v penicillin/streptomycin. The Vero cells were incubated at 37°C, 5% CO₂ for 3 days and then examined for cytopathic effect. The same protocol was used to rescue SARS-CoV virus from an infectious clone system as a control.

2.6. Confirmation of MERS-CoV virus rescue

Three techniques were used to confirm the rescue of infectious MERS-CoV (rMERS-CoV) virus from the infectious clone system. Production of infectious virions from the Vero/BHK-21 cell overlay was confirmed by triplicate blind passage of the cellular supernatant into fresh cultures of Vero Cells grown in a T150 in DMEM with 2% v/v fetal bovine serum and 1% v/v penicillin/streptomycin. Supernatant from the third blind passage was collected at 72 h post infection and rMERS-CoV RNA was extracted using the Viral RNA isolation kit (Qiagen). The viral RNA was treated with DNase I (New England Biolabs) to eliminate any plasmid contamination before being used as a template for reverse transcriptase PCR carried out with Superscript II™(Thermo Fisher) (First Strand Primer: TGTTGAGAGGCTAAGCATCTATAAGCCAAAGACACACCCGCT and Forward Primer: CAAGATGAACCTTTTGGCCTGACAAAGCGTTAGTCAATCC). The amplified cDNA was then treated with RNase H (New England Biolabs) before being removed from a containment level three laboratory (CL-3). The RT-PCR amplicon was sequenced by the Sanger method and used for phylogenetic comparison with other coronavirus sequences taken from NCBI Genbank. Stocks of the rMERS-CoV virus were frozen at −80°C degrees. These stocks were later used to compare growth kinetics of the rescued virus with the wild type MERS-CoV EMC/2012 virus.

2.7. MERS-CoV growth kinetics

Growth kinetics of the virus rescued from the infectious clone system rMERS-CoV and the clinical isolate MERS-CoV/EMC-2012

Fig 3. Supernatant from the BHK-21 and Vero cell overlay transfected with MERS-CoV/pYES1L plasmid was blind passaged three times to infect a fresh culture of Vero cells. These Vero cells were incubated for 3 days and observed for CPE. CPE was noted in the cells infected with the blind passage supernatant and not the negative control, indicating rescue of infectious virus (rMERS-CoV).
were compared by infection of Vero cells in a 12 well tissue culture plate at an MOI of 0.001. Vero Cell supernatants were collected at 24, 48 or 72 h post infection and virus titre was quantified by TCID$_{50}$ Assay.

2.8. Phylogenetic analysis

The RT-PCR amplicon of rMERS-CoV was sequenced as described in Section 2.4. The sequence was then aligned with the genomes of six other coronaviruses, downloaded from Genbank, using the MegAlign Pro software available from the DNASTAR Lasergene 11 Core Suite. The ClustalW algorithm was chosen to compute the alignment of the RT-PCR amplicon from rMERS-CoV with the corresponding sequence of: MERS-CoV/EMC-2012, BtCoV-HKU4-1, BtCoV-HKU5-1, SARS-CoV/TOR2 and CoV-229E.

2.9. Statistical methods

The error bars in Fig. 4 were generated in GraphPad Prism by calculating the standard error of the mean across the biological replicates. The resulting values were then added and subtracted from the mean value of each time point.

3. Results

3.1. Successful assembly of the MERS-CoV genome by homologous recombination in S. cerevisiae

Transformation of three MERS-CoV DNA fragments (MF1, MF2, MF3) (Fig. 1b) and the pYES1L vector into S. cerevisiae MaV203 cells yielded an assembled MERS-CoV infectious clone system. The procedure seemed quite robust as the 578 ng of input DNA produced over 200 yeast colonies (TNTC) on the selective media, the technique was performed four times and each attempt produced an intact full-length genome. This approach has also successfully constructed a variety of smaller expression plasmid constructs (Ma et al., 1987). Screening S. cerevisiae colonies by PCR with two primer sets detected the MERS-CoV genome in 22/23 samples indicating that the transformation, homologous recombination and screening processes shared a combined efficiency of approximately 95% (Fig. 2a and b). The MERS-CoV/pYES1L vector was sequenced by the Sanger method and the viral cDNA genome was 100% identical with that of the isolate MERS-CoV/EMC-2012.

3.2. Confirmation of MERS-CoV virus rescue from the infectious clone system

The utility of the MERS-CoV/pYES1L infectious clone system was examined via several techniques in BHK21 and Vero cells. Cytotoxic effect suggestive of viral infection was observed when BHK21 cells transfected with the MERS-CoV/pYES1L plasmid were overlaid onto a monolayer of Vero cells. The presence of infectious viral particles in the cellular supernatant was confirmed by three blind passages in Vero cells (Fig. 3). Viral RNA extracted from the medium of infected Vero cells was tested for MERS-CoV RNA by RT-PCR (Fig. 2c); the identity of the rescued virus was confirmed as MERS-CoV/EMC-2012 by sequencing and phylogenetic analysis of the RT-PCR amplicon (Fig. 2d). Finally, comparison of rMERS-CoV fitness to that of the clinical isolate MERS-CoV/EMC-2012 over a time course of 72 h, showed similar results. The two viruses shared replication kinetics, implying that the rescued virus serves as an accurate phenotypic surrogate for the clinical isolate (Fig. 4).

4. Discussion

Construction of infectious clone systems by homologous recombination in S. cerevisiae has potential to become the standard method for construction of coronavirus genomes or those of other similarly large and complex viruses. Use of our method avoided some of the obstacles to coronavirus genome assembly reported by other authors. For instance, regions of the coronavirus genome that show cytotoxicity in E. coli had no observed effect on the growth of S. cerevisiae. The issue of restriction site scarcity was also avoided, because unique sites were not needed to complete construction of the system. The strategy of homologous recombination seems particularly attractive when combined with the availability of viral genome sequences on Genbank and the commercial synthesis of cDNA fragments. These three resources, allow for the rapid construction of a viral infectious clone system without possession of a viral isolate or great risk of introducing mutations into a viral genome during repeated RT-PCR and PCR amplification. Combining homologous recombination in S. cerevisiae with other modern molecular methods (i.e: Gibson Assembly®) may further shorten the construction time of viral infectious clone systems (Gibson et al., 2010). Interestingly two other methods of coronavirus genome assembly (Targeted RNA recombination, and use of a Vaccinia virus backbone) both involve a variation of the homologous recombination mechanism (Almazán et al., 2014). Homologous recombination in S. cerevisiae builds upon these technologies because it allows for single-step construction of the viral cDNA genome in a BAC vector. The BAC based design does not have the stability issues of a targeted
RNA based system or require multiple steps for RNA expression like the vaccinia virus one (Enjuanes, 2005).

Two other infectious clone systems for MERS-CoV have been constructed and used to make a replication-competent propagation-deficient vaccine candidate and assess the affect of reporter gene insertion and accessory gene deletion on viral fitness (Almazán et al., 2013; Scobey et al., 2013). Many additional uses of such systems remain including further reverse genetics studies on coronavirus non-structural genes, adaptation of the viral genome to infect a suitable animal model and the creation of coronavirus vaccines (Cook et al., 2012; Stobart and Moore, 2014). Future studies will utilize our system to better understand coronavirus replicative function and potentially prepare vaccines or treatments against MERS-CoV.

5. Conclusion

The use of homologous recombination in S.cerevisiae allowed for rapid single-step construction of a MERS-CoV infectious clone system, making it a promising candidate methodology for future assembly of large and complex viral genomes or those of recently emerged zoonotic viruses.

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Contributions

A.N. A.L and B.C completed the laboratory work. D.K designed the infectious clone system, funded the project, provided expertise and edited the manuscript. D.C and S.T provided guidance, funding and edited the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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