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Research paper

Molecular and biological characterization of influenza A viruses isolated from human fecal samples

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ABSTRACT

Human influenza viruses are occasionally detected in the stools of influenza patients.

Objectives: Here, we investigated the molecular and biological characteristics of intestinal influenza viruses and their potential role in virus transmission.

Methods: Fecal samples were first screened for the presence of influenza viral RNA using RT-qPCR. Positive fecal samples were subjected to cell culture. Isolated viruses were then sequenced using MiSeq platform. Replication kinetics and receptor binding affinity were also evaluated.

Results: Influenza RNA was detected in stool samples of 41% (36/87) of influenza A positive patients. Among the 36 stool samples subjected to viral isolation, 5 showed virus growth. Sequence analysis of isolated viruses revealed two distinct mutation patterns in fecal viruses. Set I viruses was able to replicate to higher titers in cell culture despite the limited number of mutations (6 mutations) compared to set II viruses (>10 mutations). Functional analysis of both sets revealed the ability to replicate efficiently in differentiated human bronchial cells. Receptor binding testing has also demonstrated their ability to bind α 2,3 and α 2,6 sialic acid receptors.

Conclusion: The ability of fecal influenza viruses to replicate in intestinal cells and human 3D bronchial cells might suggest their possible contribution in virus transmission.

1. Introduction

Human influenza viruses primarily infect respiratory cells resulting in a wide range of respiratory illnesses (Pang et al., 2013; World Health Organisation, 2020). Typical influenza like illness (ILI) includes fever (>39 °C), muscle pain, sore throat and cough, however, severe symptoms are commonly observed among high risk groups (World Health Organisation, 2020). Further, gastrointestinal symptoms (GI) such as abdominal pain and diarrhea are observed in 23% (8%–38%) of influenza confirmed cases (Wang et al., 2003; Liou et al., 1987; Pinsky et al., 2010; Kaji et al., 2003). The avian influenza virus, H5N1, is the only influenza A virus known to replicate in human intestine and to cause severe gastrointestinal symptoms (de Jong et al., 2005; Uiprasertkul et al., 2005). Seasonal influenza viruses have been also shown to cause gastrointestinal symptoms for more than 30 years (Price et al., 1976; Peltola et al., 2003; Wootton et al., 2006). During the two epidemics in 1973 and 1974, influenza B virus was detected in hospitalized children admitted due to severe abdominal pain (Kerr et al., 1975). During the influenza A epidemic in Australia (1988), several children developed hemorrhagic gastritis of varying severity after a typical influenza-like illness (Armstrong et al., 1991). The occurrence of GI symptoms was also found to be high among patients infected with the pandemic H1N1 viruses compared to patients infected with seasonal influenza A viruses (To et al., 2010a; Tran et al., 2012; Morris et al., 2012). Several studies reported GI symptoms among patients infected with different types and/or subtypes of influenza virus (Kaji et al., 2003; Aymard et al., 2003;

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Hong et al., 2015). Despite the recognized gastrointestinal symptoms, little is known about the GI pathogenesis seen during the clinical course of influenza infections. It is possible that GI symptoms could develop due to the side effects of drug treatment, a co-infection with other enteric pathogens, or the dissemination of virus to intestinal tract.

Seasonal and pandemic influenza RNA was detected in stools of infected patients with an overall prevalence of 20.6% (Wootton et al., 2006; Tamura et al., 2010; Wootton et al., 2014; Dilantika et al., 2010; Chan et al., 2011; Yoo et al., 2010). Besides, influenza virus was occasionally isolated from stool samples using cell culture (Tamura et al., 2010; Dilantika et al., 2010; To et al., 2010b). To et al. was able to isolate viable viruses from 40% of fecal samples collected from patients with confirmed H1N1 infection (To et al., 2010b). The detection of influenza viral RNA and isolation of viable influenza viruses from fecal samples of infected patients suggest the localization of virus in patients' intestinal tract; and hence may serve as a potential mode of transmission during influenza outbreaks.

The spread of influenza virus to the GI tract is thought to occur after a primary respiratory infection, yet, the route of dissemination remains unknown. Current knowledge attributes the detection of influenza viruses in feces to (i) swallowing of influenza viruses from respiratory secretions; (ii) remnants of infected submucosal intestinal antigen-presenting immune cells; and (iii) virus replication in intestinal cells (Minodier et al., 2015). The ability of seasonal influenza viruses to bind to influenza virus sialic acid receptors in the human gastrointestinal tract, and to replicate within these cells is still debatable. Finne et al. and Sata et al. did not find any evidence for α 2,6-linked sialic acid, the primary human influenza virus receptor, on mucosa of the colon or small intestine (Finne et al., 1989; Sata et al., 1991; Yao et al., 2008). Nevertheless, this binding specificity is not absolute (Stevens et al., 2006), and α 2,3-linked sialic acids are abundantly expressed on colorectal epithelial cells (Sata et al., 1991).

The significance of the detection of influenza viral RNA in stools, their spread to GI tract, their ability to replicate in intestinal cells, and the potential contribution of execrated viable viruses in infection transmission remain unclear and are debated (Kocer et al., 2013). In the present study, we aim to (i) investigate the presence of seasonal influenza viruses in the stools of adult patients presenting influenza like illness, regardless of GI symptoms; (ii) examine the association of respiratory viral load with the shedding of influenza RNA in stools and/or isolation of viable viruses; (iii) explore the molecular characterization and diversity of fecal influenza viruses and their possible role in transmission.

2. Material and methods

2.1. Study population and sample collection

Samples were collected from adult patients presenting at emergency department with ILI during winter seasons of 2018 and 2019. Patients were included if they sought care within 1 week of illness onset. After providing written, informed consent, patients were asked to submit nasal and stool specimens regardless of gastrointestinal symptoms. Upon collection, nasal swabs were placed in 2–3 ml of viral transport media (VTM) and stored at −80 °C. Fecal samples were resuspended in a 10% phosphate buffered saline (PBS), centrifuged at 4000 rpm for 20 min. Supernatants were collected, aliquoted and kept at −80 °C.

2.2. Influenza virus RNA extraction, detection and quantification

Viral RNA was extracted from 140 μl of VTM using the QIAamp viral RNA mini kit as instructed by manufacturer (Qiagen, Germany). For fecal samples, viral RNA was extracted from 200 μl of filtered (0.22 μm filters) fecal suspension using AllPrep PowerViral DNA/RNA kit (Qiagen, Germany) according to manufacturer's instructions. All extracted RNA samples were screened for influenza by one-step RT-qPCR. Influenza A virus-positive nasal samples were subtyped as previously described in the protocols developed by World Health Organization (World Health Organisation, 2017). For virus quantification, a standard curve relating copy number to Cq value was generated based on 10-fold dilutions of a control plasmid run in triplicate. All statistical analysis was performed using Prism 7.

2.3. Isolation of influenza virus from fecal samples

Influenza-positive fecal samples were selected for cell culture isolation in human colorectal cancer cells (caco-2). Samples were filtered twice using 0.22 μm filters before being added on confluent caco-2 cells cultured in infection medium (IM; MEM medium, 0.3% bovine serum albumin (BSA), and 1 μg/μl TPCK-trypsin). Cells were then incubated at 37 °C for 7 days. At the end of this period or when the cytopathic effect (CPE) was seen, supernatants were collected and tested for the presence of influenza A using real-time PCR. Negative samples underwent a maximum of three passages.

2.4. Deep sequencing of isolated influenza viruses

Full genome amplification and sequencing of viruses isolated from fecal samples was performed as previously described (Al Khatib et al., 2020). In brief, the coding region of the whole influenza genome was amplified using the Superscript III one-step RT-PCR Platinum Taq HiFi kit (Invitrogen, USA) and influenza-specific primers as described by Zhou et al., 2009 (Yao et al., 2008). PCR products were quantified and diluted to 0.2 ng/μL as recommended by the Nextera XT library preparation kit (Illumina, USA). DNA libaraies were prepared according to the manufacturer's instructions. Briefly, diluted PCR products were fragmented and tagged with the Nextera XT adapters. Fragmented PCR products were then amplified by a 12-cycle PCR program to add the indexes. Amplified indexed fragments were purified and size-selected using Agencourt AMpure XP beads (Beckman Coulter, USA). Equal nanomolar concentrations of normalized libraries were pooled and diluted to a final concentration of 8 pM. Libraries were added to a MiSeq 300-cycle reagent cartridge. All samples were run in duplicates starting from RT-PCR step. Plasmid controls for H1 and H3 were included as well. For variant analysis, only mutations detected in both duplicates were considered valid and were analyzed. Plasmid sequences served as internal controls to improve the accuracy of variant identification. All mutations reported in plasmid were excluded from the analysis and considered PCR errors. Viruses from nasal samples were PCR amplified as indicated above and sequenced using sanger sequencing technology. All were deposited in the NCBI (accession numbers are listed in Supplementary Table 1).

2.5. Data processing and variant calling

Sequencing reads were filtered to remove low quality (Phred score < 30) and short reads (<50 nt) using Trim-Galore tool v1.33 (Krueger, 2015). Filtered reads were then mapped to the pH1N1 (EPI_ISL_227813) and H3N2 (EPI_ISL_233740) reference genomes using the default settings of the Burrow-wheeler aligner (BWA) (Li and Durbin, 2009). Consensus sequences were constructed using VCFtools (Danecek et al., 2011). Variant calling was done at both concesus (frequency > 90%) and sub-consensus (frequency 5–50%) levels using GATK analysis toolkits (McKenna et al., 2010). A minimum of 100× coverage was used to call variants at consensus sequence level and a1000x coverage to call variants at sub-consensus sequence level (frequency 5–50%). For the later, reads were mapped against the consensus sequence of each sample.

2.6. Cell line

Madin-Darby canine kidney cells (MDCK) and Caco-2 cells were maintained in DMEM medium (Gibco, USA) supplemented with penicillin-streptomycin and 7% heat-inactivated fetal bovine serum (HI-FBS; Gibco, USA). Differentiated human bronchial/tracheal cells (HBT; Lifeline, USA) were grown and maintained under air-liquid interface (ALI) conditions according to the manufacturer's recommendations. Briefly, cells were plated at a density of 50,000 cells per well on permeable transwell (6.5-mm diameter) supports coated with 0.3 mg/ml growth factor reduced matrigel (Life Technologies, USA). Once confluent, medium was removed from the apical surface while differentiation medium (Lifeline, USA) was added to the basal chamber. HBT cells were maintained in ALI for 35 days to form polarized cultures that resemble *in vivo* pseudostratified mucociliary epithelium. Differentiation of HBT cells was confirmed by visualizing cells with scanning electron microscopy (SEM) (Supplementary Fig. 1).

2.7. Quantification of virus using TCID50 assay

Viral titers of isolated influenza viruses were estimated using 50% tissue culture infective dose (TCID50) assay. MDCK cells were seeded on 96-well plates and infected the following day for 2 h with 10 log dilutions of virus in IM. Positive and negative virus controls were used to evaluate CPE. Virus inoculum was removed and replaced with IM. Cells were incubated at 37 °C for 3 days. The presence of CPE was examined, and $TCID_{50}$/0.1 ml values were calculated using the Reed-Muench method formula (Reed and Muench, 1938). Known positive and negative samples were used as controls.

2.8. Functional analysis of intestinal influenza viruses: Replication kinetics

To test the ability of fecal viruses to replicate in human bronchial cells, we cultured human primary bronchial and colon cells in 48-well plate. Once confluent, viruses (from original fecal samples) were added, incubated for one hour at 37 °C. Known positive and negative samples -of nasal origin- were also used as controls. Viral inoculum was then removed and replaced with IM. Supernatant was collected at times 0, 24, 48, 72- and 96-h post infection (hpi). RNA was extracted and RRT-PCR was used for quantification. Viral titers in the cell culture supernatants were assayed by quantitative real-time RT-PCR.

2.9. Functional analysis of intestinal influenza viruses: Receptor binding

To test the ability of fecal viruses to bind α 2,3 and α 2,6 SAG receptors, we used a competitive binding assay. The assay was optimized using different concentrations of lectins to determine the optimal concentration required to block α2,6 and α2,3 receptors on cells (Supplementary Fig. 2). MDCK cells were seeded in 96-well plate. Once confluent, cells were washed with TPBS (PBS + 0.05% Tween™ 20) and fixed with 4% paraformaldehyde for 30 min at room temperature. Non-Specific binding was blocked by incubating cells with 0.2% BSA in PBS for 2 h at 37 °C. After washing with TPBS, biotinylated SNL (20 μg/ml) and MAL (30 μg/ml) lectins (Vector laboratories, USA) were added to the separate wells and incubated for 30 min at room temperature. Positive H3N2 controls as well as isolated viruses were then added to wells and incubated at 4 °C for one hour. Wells not coated with cells but blocked and treated with virus as indicated above were used as negative controls. Cells with SNA and MAA were also used as controls to calculate OD. Anti-H3 antibody was then added to wells for 30 min followed by additional 30 min incubation with peroxidase labeled anti-H3 antibody. A substrate was then applied to all wells and the developed color was quantify by spectrophotometry. The optical density of each well was calculated as follows:

2.10. Infection of human bronchial/tracheal cells

Viruses isolated in caco-2 cells were diluted in human bronchial cells basal medium to equal titers as determined by TCID50 assay. HBT cells were washed to remove excess mucus secretion on the apical surface prior to infection with MOI of 0.1 of influenza virus in a 100 μl inoculum. Cells were infected as indicated. Viruses released apically were harvested by adding 200 μl of 0.05% BSA in bronchial basal medium at different time points: 24 h, 48 h, 72 h, 96 h, 120 h and 144 h), allowed to equilibrate at 37 °C for 30 min, then collected. Viral titers in the cell culture supernatants were assayed by quantitative real-time RT-PCR. All infections were done in duplicates.

3. Results

3.1. Prevalence of influenza virus in the stools of influenza infected patients

A total of 222 adults with ILI were recruited during the study period extending from January 2018 to May 2019. Median age of patients was 32 years (17–68 years). Seasonal influenza viruses were detected in 51% ($n = 114$ patients) of nasal samples. Influenza viral RNA was detected in 37.7% (43 of 114) of stool samples. The majority (84%) of viral RNA detected in stool samples belongs to influenza A type (Fig. 1A). The prevalence of influenza viral RNA varied among patients of various ages. Fecal RNA shedding rates were high among teens (25%) and in patients who are in their thirties (19%) compared to lower shedding rates in patients older than 40 years old (Fig. 1B). Overall, the mean fecal viral RNA concentration was $6.5 \pm 2.7 \times 10^4$ copies/ml of fecal suspension and the median was 1.2×10^4 copies/ml of fecal suspension (Fig. 2A). As expected, all patients had higher nasal viral concentrations compared to fecal virus concentrations, except for six patients who showed 2 to 10-folds increase in virus concentration in their fecal samples (Fig. 2B). In this study, fecal excretion of virus was not correlated with the appearance of gastrointestinal symptoms. Only four patients simultaneously presented diarrhea/vomiting and fecal excretion of influenza virus. All fecal samples which belong to patients with positive respiratory results were subjected to viral isolation in cell culture. From 87 stool specimens tested, 10 samples were found positive after 2 to 3 passages in caco-2 cells. A significant corrolation ($p < 0.001$) was observed between fecal viral load and positive isolation of viruses in caco-2 cells. Interestingly, all isolated viruses were H3N2 viruses; one of which belong to patients with confirmed mixed H1N1 and H3N2 nasal infection.

3.2. Molecular characterization of intestinal influenza viruses

To investigate genetic diversity of fecal viruses, we performed deep genome sequencing of viruses isolated in caco-2 cells. Only 5 (out of 10) viruses were successfully sequenced. A total of 59 synonymous mutations were detected in HA sequences of these five samples. Analysis of HA sequences at amino acid level revealed two patterns of mutation clusters as compared to corresponding nasal samples and 2016–2018 vaccine strain (A/Hong Kong/4801/2014; EPI_ISL_233740) (Fig. 3 and Supplementary Fig. 3). Set I which includes samples F-129 and F-134 exhibited 98.94% and 99.47% similarity to A/Hong Kong/4801/2014 (H3N2); respectively. Set II samples (F-120, F-195 and F-203), on the other hand, showed 96.1% similarity to A/Hong Kong/4801/2014; but more than 99.1% similarity to the old H3N2 vaccine strain, A/Brisbane/10/2007. Identified mutations were distributed differently across HA protein of viruses belonging to each set (Fig. 3A). HA sequences of set I viruses exhibited two substitutions in HA head domain: T160K and L194P (Fig. 3B). All three mutations are commonly detected (>75%) in HA sequences deposited in GISAID database since January 2017 (n = 17,075 sequences; Supplementary Table 2). HA sequence of F-134 sample had three additional substitutions: R142K, M168V and Q197R. Unlike other mutations, these substitutions were not commonly reported among H3N2 strains sequenced worldwide (Supplementary Table 2). R142K and Q197R in A3 and B2 antigenic sites were reported in 24% and 6.5% of HA sequences, respectively, while M168V was found in less than 0.5% of HA sequences. Mutation pattern among set II samples was

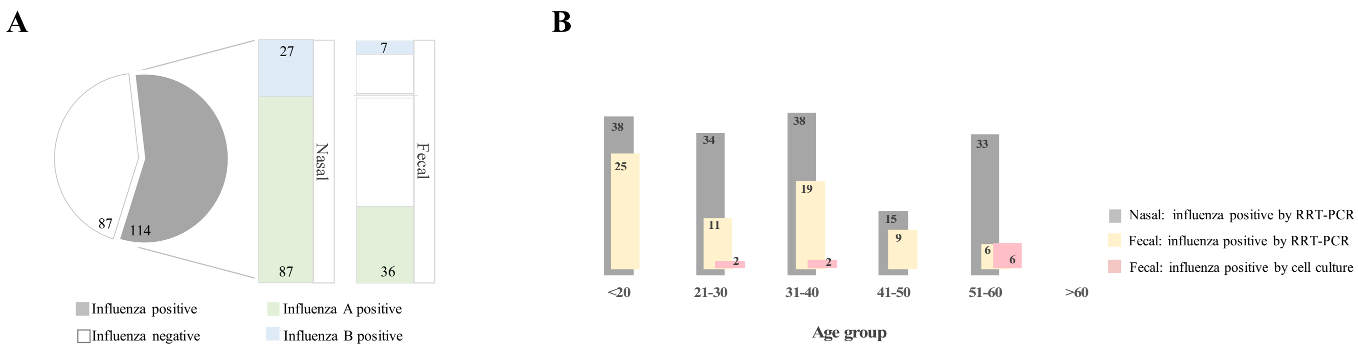

Fig. 1. Detection of influenza viruses in nasal and fecal samples of patients with flu like illness. (A) Prevalence of influenza A and B viruses in nasal and fecal samples of 114 influenza patients. (B) Percentage of patients in different age groups who tested positive for influenza in their nasal and/or fecal samples.

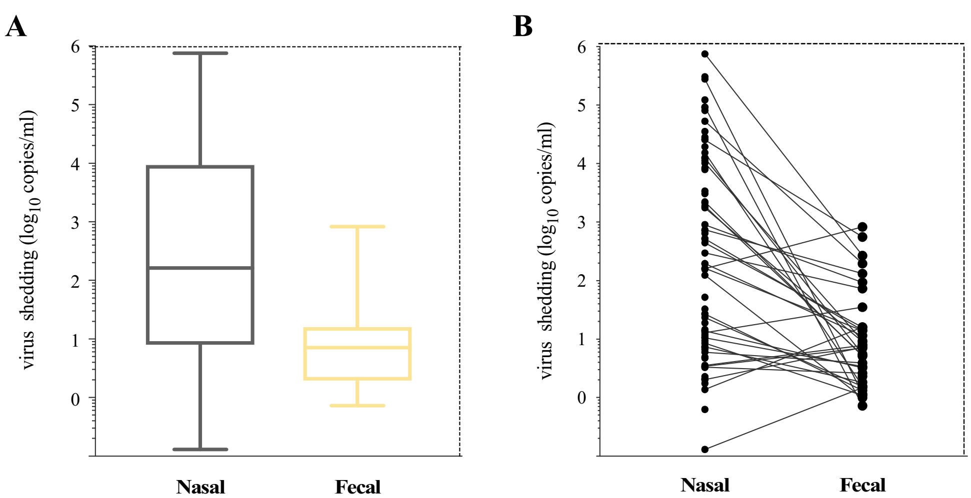

Fig. 2. Influenza virus shedding levels in patients with confirmed influenza infection. (A) Viral RNA shedding rates -expressed as $\log_{10}$ copies/ml- in nasal ($n = 87$) and fecal ($n = 36$) samples of all influenza positive patients. (B) Comparison of viral RNA shedding rates in nasal and fecal samples of influenza positive patients.

identical; with a total of 21 mutations as compared to A/Hong Kong/4801/2014 (Fig. 3B). Notably, most of these mutations were rarely reported among H3N2 HA sequences after 2017 (Supplementary Table 2). Based on mutation-site analysis, seven of set II mutations are located within RBS; five of which are also found in antibody recognition sites (Supplementary Table 2). Mutation A138S, in particular, has been linked to virulence and host specificity shift (Busch et al., 2008). The rest of mutations ($n = 12$ mutations) appeared in the HA stem domain (Fig. 3A). Analysis of genetic diversity of isolated fecal viruses at sub-consensus level revealed limited genetic diversity with only one haplotype detected in all samples.

Analysis of NA sequences of fecal viruses isolated in caco-2 cells has also revealed similar patterns of mutation clustering among samples within each set (Fig. 4). In total, 41 mutations were detected in NA sequences of viruses isolated from fecal samples samples. Mutations identified in set I samples were less in number and more prevalent as compared to global N2 sequences. A total of seven mutations were detected in NA sequences of set I viruses, two of which (V149A and S247T) are associated with decreases sensitivity to neuraminidase inhibitors (NAIs). In contrast, mutations of set II viruses were more in number and less prevalent globally. Set II viruses were carrying 10 mutations including R150H mutation that is linked to strong NAI resistance.

Analysis of NP and M genes of all viruses revealed a limited number of mutations (Supplementary Fig. 4). Mutation analysis of NS protein, on the other hand, showed the presence of eight mutations. Two of these mutations (N74D, V182I) were reported in all fecal viruses. With the exception of these two mutations, all NS mutations were rarely reported among H3N2 viruses circulating worldwide.

3.3. Biological characterization of intestinal influenza viruses

Here, we evaluated the replication and receptor binding properties of isolated fecal viruses. First, we compared the replication efficiency of isolated viruses in human respiratory and intestinal cells (Fig. 5). Viruses that belong to the same set behaved differently in terms of replication in both cell lines. Set I viruses, for example, which share more than 99.5% of their genome sequences showed opposite replication patterns in undifferentiated bronchial cells. Viruses isolated from F-134 sample replicated to high titers in human bronchial cells while viruses in F-129 samples showed weak replication that peaked at 48 hpi and declined at later time point. Similarly, viruses isolated from F-120 sample replicated weakly compared to viruses of the same set (Fig. 5A). Compared to bronchial cells, isolated viruses demonstrated very weak replication in colon cells particularly set II viruses which were barely detectable at the end of incubation period (72 hpi) (Fig. 5B). Altogether, these findings suggest that these viruses are probably passing through gastrointestinal tract rather than replicating there. However, the ability of viruses to replicate in other intestinal cell types should be investigated.

Differences in replication patterns could be attributed to receptor binding capabilities of isolated viruses. Overall, fecal viruses showed increased binding to α2,3 sialic acid receptors (Hadfield et al., 2018) compared to α2,6 SAG receptors (Fig. 6) regardless of virus set. However, there was no significant differences in binding levels. As a control,

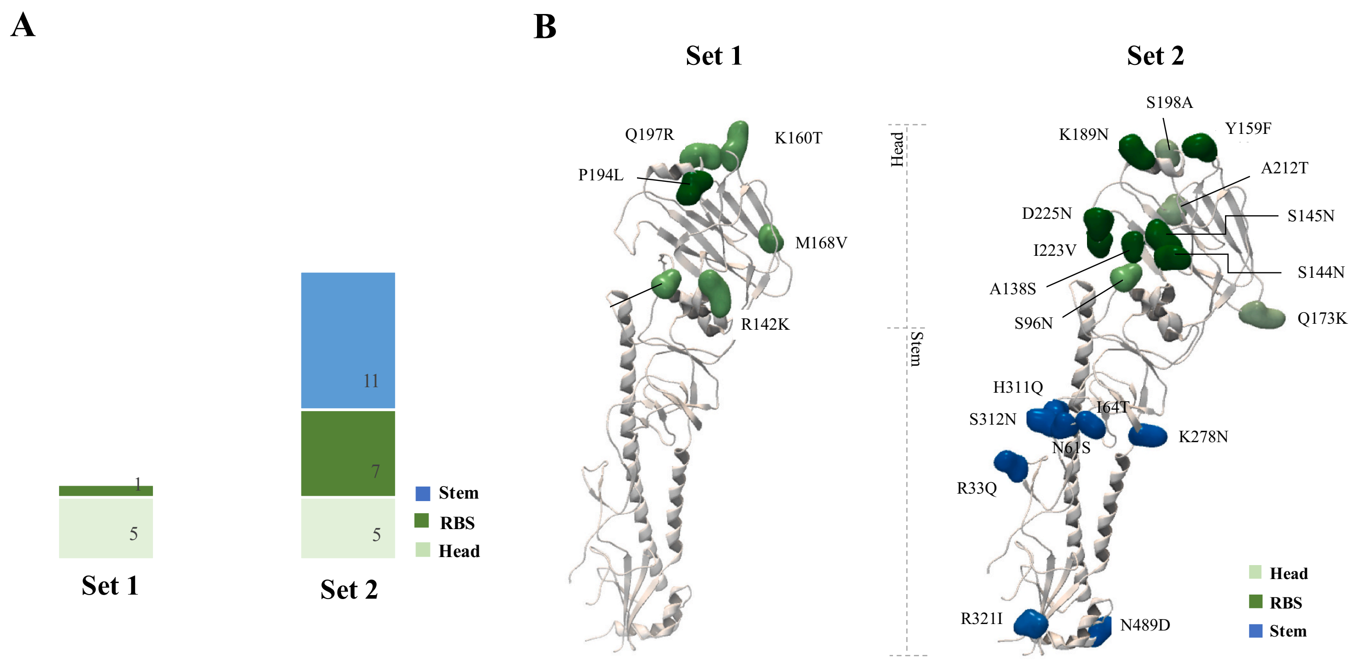

Fig. 3. Distribution of amino acid mutations in HA protein of isolated fecal influenza viruses. (A) Bars represents number of substitutions identified in head domain (light green), receptor binding site (RBS; dark green) and stem domain (blue) of HA protein in set I and set II fecal influenza viruses. (B) Three dimentional (3D) structure of influenza virus HA monomer (PDB 2YP7) displaying mutations detected in HA protein of fecal influenza viruses isolated in caco-2 cells and differentiated HBT cells.

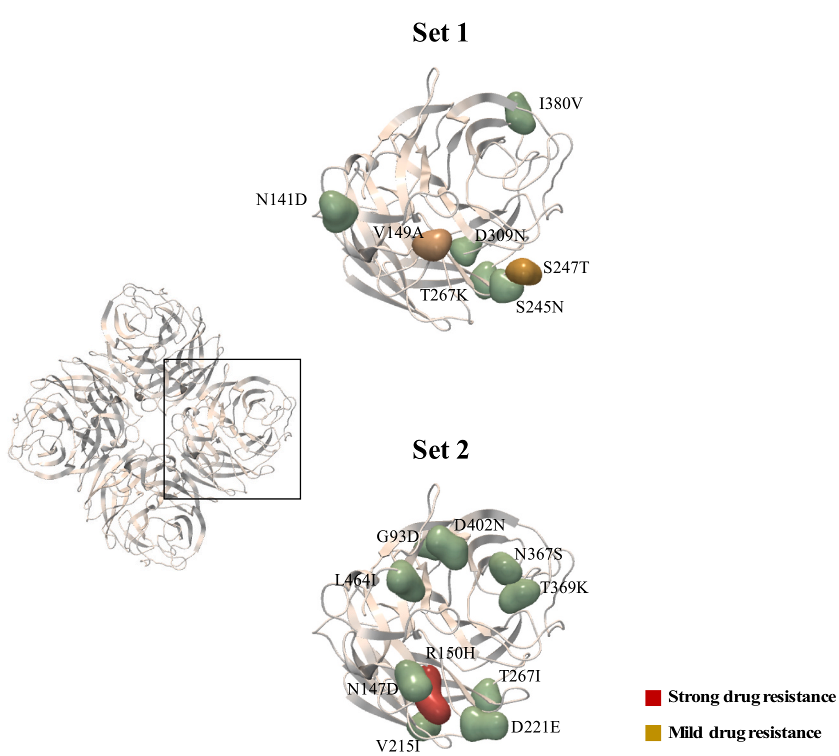

Fig. 4. Distribuation of amino acid mutations in NA protein of fecal influenza viruses. Three dimentional structure (3D) of NA monomer (PDB 4GZX) showing amino acid mutations in NA protein of influenza viruses isolated from fecal samples using caco-2 cells.

H3N2 virus of nasal origin was included from one of nasal samples that had a negative stool result (N-NS). The control virus showed preferential binding to α2,3 SAG. In contrast, H3N2 viruses in nasal samples of 120 and 195 showed increased binding levels to α2,3 SAG similar to their fecal viruses' counterparts.

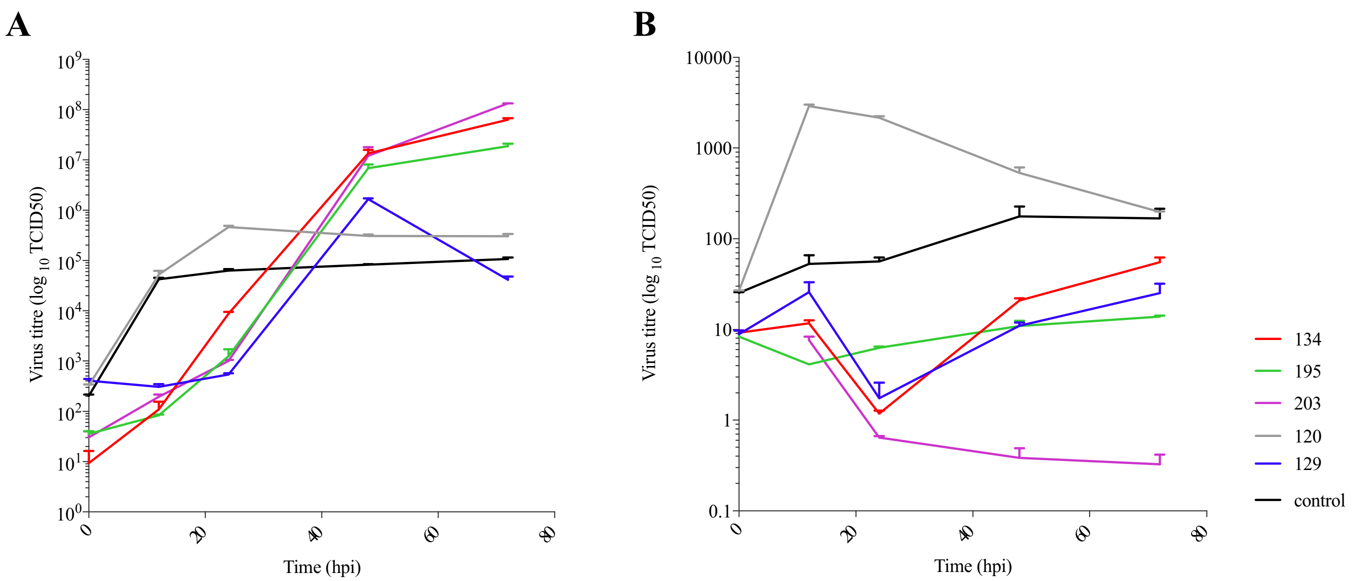

Fig. 5. Replication of fecal influenza viruses in primary human bronchial and colon cells. Human primary bronchial (A) and colonic (B) epithelial cells were infected with isolated viruses. Virus titers were measured at indicated time points (x-axis). H3N2 viruses from nasal sample was used as a control.

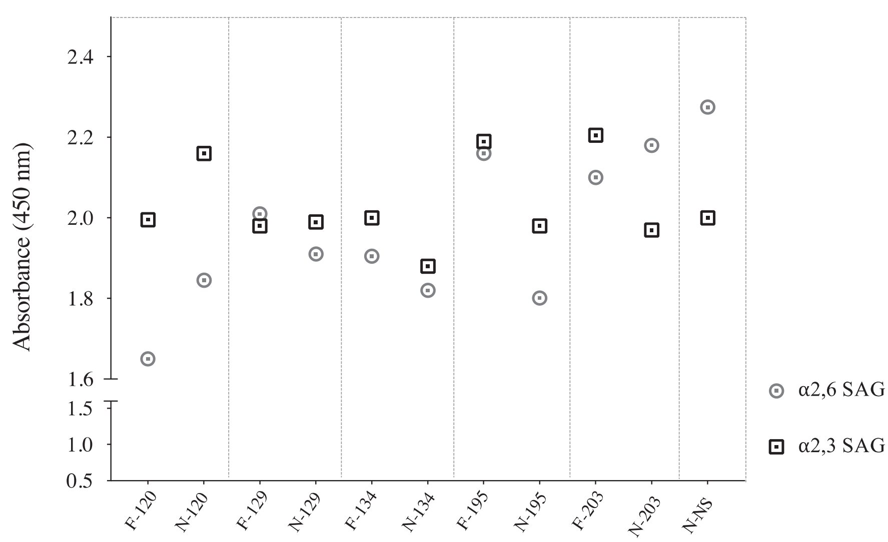

Fig. 6. Binding of fecal influenza viruses to α2,6- and α2,3- SAG of MDCK cells. Isolated fecal Viruses showed the ability to bind both receptors; however, with increased binding levels to α2,3 SAG. Corresponding nasal samples were also included. N-NS (positive nasal–negative fecal sample) was used as a control.

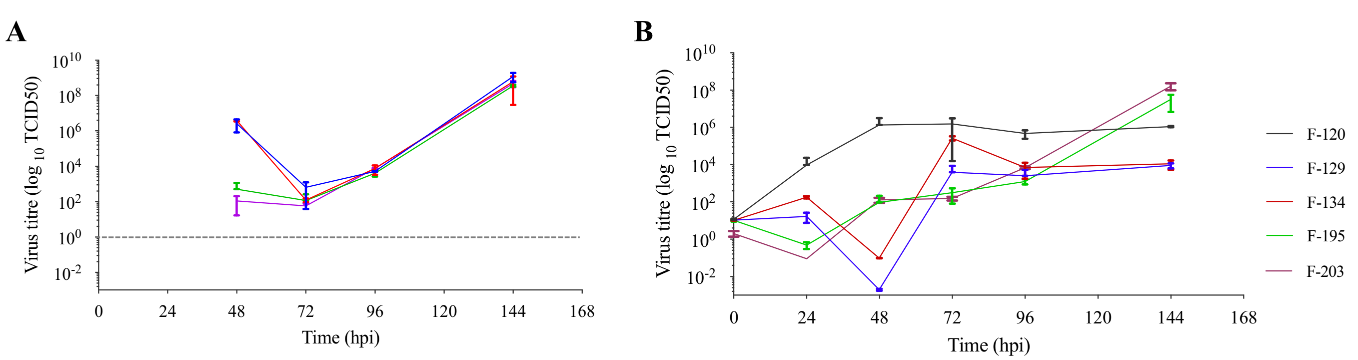

Fig. 7. Replication of fecal influenza viruses in differentiated human tracheal/bronchial epithelial (HBT) cells. Both the original fecal viruses (A) and caco-2-isolated viruses (B) replicated efficiently in differentiated HBT cells. Viruses from original fecal samples demonstrated a gradual increase in virus titer after the third day of infection regardless of virus HA set (A); while replication patterns were more consistent among samples of the same set in caco-2 isolate viruses (B).

3.4. Intestinal influenza viruses replicate efficiently in differentiated human airways cells

The shedding of viable viruses in stools of infected patients may suggest a possible role in virus transmission. To assess the ability of fecal influenza viruses to replicate in human respiratory tract, fecal influenza viruses were applied on differentiated HBT cells. Fully differentiated HBT cells were infected with viruses isolated in caco-2 cells as well as viruses from original samples. Replication results demonstrated similar rerplication patterns of all fecal viruses in human bronchial/tracheal cells regardless of their mutation pattern (Fig. 7A). In contrast, the replication kinetics of viruses isolated in caco-2 cells were similar among viruses that belong to the same set (Fig. 7B). Set I viruses deminstarted a significant reduction in the replication at 48 hpi. After 72 hpi, the two viruses showed minimal increase in the replication that remained the same until 144 hpi. The replication of set II viruses, on the other hand, continued to increase gradually over time (Fig. 7B). This might be related to differences in mutation patterns between set I and set II viruses. To further confirm the link between observed replication kinetics and mutation patterns, we performed deep sequencing analysis of viruses collected after one cycle of virus infection in HBT cells. HA sequence analysis showed similar mutation clustering patterns as indicated above in viruses isolated in caco-2 cells. Deep sequence analysis has also revealed the presence of only one haplotype in all samples. This may indicate the high fitness of these viruses in HBT cells, which reduced further cell-specific mutations. Most importantly, this provides a preliminary evidence of the ability of fecally-excreted influenza viruses to re-infect human respiratory tract and the possibility of fecal-oral transmission.

4. Discussion

The primary target of human influenza viruses is the respiratory tract which is also considered to be the main route of transmission. However, extrapulmonary symptoms are commonly observed in patients during the course of infection. About one third of influenza patients show gastrointestinal symptoms which may be a sign of severe infection in many cases (Kaji et al., 2003; Kerr et al., 1975; Morris et al., 2012; To et al., 2010b; Wie et al., 2013). Further, viral RNA, and occasionally viable viruses, have been detected in stools of patients with confirmed influenza infection (Wootton et al., 2006; Tamura et al., 2010; Wootton et al., 2014; Dilantika et al., 2010; To et al., 2010b). These findings raise many questions about the origin of virus, their ability to replicate in intestinal cells and their possible contribution to virus transmission (Tamura et al., 2010; Dilantika et al., 2010; To et al., 2010b).

In this study, we investigated the occurrence of fecal influenza in patients with confirmed respiratory influenza infection. Positive fecal influenza was reported in 38% of patients with an overall lower shedding rates compared to their nasal sample counterparts. Only three fecal samples displayed higher viral titers compared to nasal samples. Studies reporting fecal viral shedding among infected patients found that the overall rates ranged from 7 to 47% in adults (Dilantika et al., 2010). Similar to our findings, the prevalence of influenza RNA shedding is higher in younger patients (3 to 71%) (Minodier et al., 2015; Chan et al., 2009).

The detection of influenza RNA in fecal samples doesn't not accurately reflect the shedding of viable virus that can initiate a new infection. Viral stool cultures of influenza patients are not commonly ordered, therefore, the true occurrence of viable influenza virus in stool is unknown. Some studies, although rare, have tried to isolate viable viruses in cell cultures (Wootton et al., 2006; Tamura et al., 2010; Chan et al., 2011). Three of these studies were able to isolate intact fecal viruses (Tamura et al., 2010; Dilantika et al., 2010; To et al., 2010b). Here, we were able to isolate viable viruses after limited number of passages in caco-2 cells as well as in human bronchial/tracheal cells. The low yield of influenza virus in stool samples could be related to the presence of inhibitory materials in the stool. It could also be attributed to waiting time between collection and transportation. The origin of fecal influenza virus and the mechanism by which it spreads to GI tract and survive in its environment are poorly described. Current knowledge explains the detection of human influenza viruses in feces by the swallowing of influenza viruses from the upper respiratory tract and/or the replication of virus in intestinal cells (Minodier et al., 2015). Here, we hypothesized that swallowed influenza viruses undergo a strong selection for genetic variants that can support their capabilities to survive GI environment or even replicate in intestinal cells. Therefore, we performed deep sequencing of isolated fecal viruses to explore the molecular characterization of shedded viruses. To our best knowledge, this is the first paper that described molecular characterization of fecal viruses. As expected, majority of mutations were detected in HA gene. Set I viruses had limited number of mutations, yet one-third of mutations identified were located in receptor binding domain which may be a sign of adaptation to intestinal receptors. The limited mutations in set I viruses may also indicate their ability to rapidly adapt to intestinal cells and/or to survive there. A study in mice model have previously shown that fecal isolates were carrying novel HA mutations that were associated with severe symptoms (Kocer et al., 2013).

The presence of intact influenza viruses in stool samples could be attributed to virus ability to bind SA receptors on intestinal cells. Human influenza A viruses preferentially bind α2,6 SA receptors but can also bind 'avian-like' α2,3 SA receptors (Shinya and Kawaoka, 2006; Couceiro et al., 1993). Shu et al. have previously confirmed the abundant expression of α2,6 SA receptors on GI epithelial cells (Shu et al., 2010). Examination of human intestinal cells have also revealed the presence of α-2,3 SA receptors on cells of ileum to the rectum, with abundant expression in goblet cells mostly found in the large intestine (Shu et al., 2010). Here, we found that fecal viruses of both sets were able to bind both forms of SA receptors, however, binding levels to α2,3 SA were generally higher for all viruses. The ability of fecal viruses to bind both types of cells suggest that fecal viruses have the potential to infect and replicate in human intestinal epithelial cells. Hirose et al. (2016) provided an evidence of the active replication of influenza A viruses in patients with colitis (Hirose et al., 2016). However, we have observed a very weak -if at all- replication of fecal viruses in human colonic epithelial cells compared high replication rates in bronchial, caco2 and MDCK cells.

Several human respiratory viruses of zoonotic origin, such as SARS-CoV, MERS-CoV and influenza viruses were detected in stools of infected patients. Still, there is no evidence of fecal-oral transmission for any (Chan et al., 2009; Corman et al., 2016; Cheng et al., 2004). Here, we explored the ability of shedded viruses to cause respiratory infection and observed efficient replication in human bronchial/tracheal 3D cells. This finding may signify the ability of fecal influenza viruses to initiate respiratory infection *via* fecal oral transmission, regardless of their ability to replicate in intestine.

5. Conclusion

Influenza viruses infect cells through binding to sialic acid receptors which are commonly expressed in many human cell tissues. Here, we investigated the extrapulmonary replication of influenza viruses in intestinal cells and their potential role in transmission. Our findings showed that fecal shedding occurs in about 30% of influenza patients, 7% were shedding viable influenza viruses that had specific molecular and biological characteristics. Importantly, these viruses demonstrated the ability to replicate in human bronchial/tracheal 3D cells suggesting a role in spread the infection. Additional studies of large prospective cohorts may provide further insights into the role of human influenza viruses in the intestinal system. Understanding the mechanisms of fecal influenza virus shedding might provide further information to help in understanding virus evolution, cell tropism, pathogenicity and transmission dynamics for designing management policies.

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Ethical approval

Ethical approval of this study was obtained from institutional review board committees at Qatar University (QU-IRB-903-E/18) and Hamad Medical Corporation (16335/16).

Authors contribution

H.M.Y. originated the idea, H.M.Y. and H.A.K. designed the experiments, H.A.K. performed the experiments and ran the analysis, H.M.Y., M.A.M., & A.A.T. obtained the funding for this study, P.V.C. and S·P facilitated samples and clinical data collection. H.A.K. wrote the original draft of the manuscript, H.M.Y. revised it, and all the authors agreed on the final version before submission.

Declaration of Competing Interest

Authors declare no conflict of interest.

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