Clinical Microbiology and Infection 22 (2016) 813.e1–813.e7

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Long-term detection of seasonal influenza RNA in faeces and intestine

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ARTICLE INFO

Article history:
Received 27 April 2016
Received in revised form
17 June 2016
Accepted 22 June 2016
Available online 15 July 2016

Editor: L. Kaiser

Keywords:
Colitis
Colonoscopy
Faeces
Influenza
Intestine

ABSTRACT

Some cases of seasonal influenza virus (human influenza A virus (IAV)/human influenza B virus (IBV)) are associated with abdominal symptoms. Although virus RNA has been detected in faeces, intestinal infection has not been clearly demonstrated. We aimed to provide evidence that IAV/IBV infects the human intestine. This prospective observational study measured virus RNA in faecal and sputum samples from 22 patients infected with IAV/IBV (19 IAV positive and three IBV positive). Nineteen patients were included in the analysis and were assigned to faecal IAV-positive and -negative groups. Virus kinetics were examined in faecal samples from an IAV-infected patient (patient 1) and an IBV-infected patient (patient 2). Finally, intestinal tissue from an IAV-diagnosed patient who developed haemorrhagic colitis and underwent colonoscopy was examined for the presence of replicating IAV (patient 3). Virus RNA was detected in faecal samples from 8/22 IAV/IBV-infected patients (36.4%). Diarrhoea occurred significantly more often in the faecal IAV-positive group (p 0.002). In patients 1 and 2, virus RNA became undetectable in sputum on days 7 and 10 after infection, respectively, but was detected in faeces for a further 2 weeks. Virus mRNA and antigens were detected in intestinal tissues (mucosal epithelium of the sigmoid colon) from patient 3. These findings suggest that IAV/IBV infects within the intestinal tract; thus, the human intestine may be an additional target organ for IAV/IBV infection. **R. Hirose, CMI 2016;22:813.e1–813.e7**

Introduction

Most patients infected with seasonal influenza virus (human influenza A virus (IAV)/human influenza B virus (IBV)) show both upper respiratory symptoms (cough and throat pain) and general symptoms (fever, fatigue, headache and muscle pain) [1,2]; however, some also experience abdominal pain, vomiting and diarrhoea [3–5]. Usually IAV/IBV-infected patients expel virus-containing sputum to expel the pathogen from the airway; however, some patients, especially the elderly or infants, swallow virus-contaminated sputum. Therefore, we hypothesized that abdominal symptoms may be associated with IAV/IBV infection of the gastrointestinal tract.

Previous retrospective studies detected IAV/IBV RNA in faecal samples from individuals diagnosed with infectious enteritis after experiencing upper respiratory symptoms caused by the virus [6–8]. A prospective observational study detected IAV RNA in faecal samples from adults hospitalized with laboratory-confirmed IAV infection [9]. RNA derived from the pandemic H1N1/09 strain was detected in faeces from a patient infected with this strain, and in some cases infectious virions were isolated from faecal samples [10–12].

These findings support our hypothesis that IAV/IBV infects human intestinal tissue *in vivo*. Although these reports suggest possible IAV/IBV infection of the human intestine, it is unclear whether IAV/IBV infects and replicates in the human intestinal tract *in vivo*. Limited information about intestinal infection by IAV/IBV may lead to diagnostic errors, e.g. patients infected with IAV/IBV

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http://dx.doi.org/10.1016/j.cmi.2016.06.015
1198-743X/

misdiagnosed as having enteritis caused by other pathogens. Such diagnostic errors result not only in inadequate treatment but also in the spread of viral infection. Therefore, it is clinically important to provide definitive *in vivo* evidence that IAV/IBV infects the human intestine.

The aim of this study was to provide evidence to support the hypothesis that IAV/IBV infects the human intestine. We focused on virus kinetics in faecal samples from IAV/IBV-infected patients. We also tried to detect persistent release of the virus genome in faecal samples from these patients.

Methods

Cells and reagents

Madin-Darby canine kidney cells (MDCK) were purchased from the Riken BioResource Center Cell Bank (Ibaragi, Japan) and cultured in minimal essential medium (Sigma, St Louis, MO) supplemented with 10% foetal bovine serum and standard antibiotics.

Virus RNA detection and virus isolation from clinical specimens

Sputum was centrifuged at 3000*g* for 5 minutes to remove cell debris, and the supernatant was collected. Faecal samples were dissolved in phosphate-buffered saline and centrifuged at 3000*g* for 5 minutes, and the supernatant was collected. The supernatants from the sputum and faecal samples were analysed for virus RNA using quantitative real-time PCR (qRT-PCR) as described below.

MDCK cells were seeded into 12-well plates at a density of 5×10^5 cells per well. Supernatants obtained after centrifugation were sterilized by passage through a 0.22 μm filter, and 200 μL was inoculated onto MDCK cells to isolate infectious virions. MDCK cells were cultured at 33°C in Dulbecco modified Eagle medium/F-12 (Thermo Fisher, Waltham, MA) containing 0.2% bovine serum albumin and trypsin (3 μg/mL).

The sputum and faecal samples were stocked and analysed separately to prevent cross-contamination.

RNA extraction and qRT-PCR

Nucleic acid was extracted from samples using the PureLink RNA Mini Kit (Thermo Fisher), and cDNA was prepared using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). IAV was detected and quantified using RT-PCR and Thunderbird SYBR qPCR Mix (Toyobo). Virus RNA (genomic RNA/mRNA) and mRNA was quantified individually after cDNA was synthesized using a random/oligo dt primer (virus RNA detection) or an oligo dt primer (virus mRNA detection). IAV-specific PCR was performed using an IAV matrix protein gene-specific primer set (M30F: 5′-TTCTAACCGAGGTCGAAACG-3′ and M264R2: 5′-ACAAAGCGTCTACGCTGCAG-3′) designed by the National Institute of Infectious Diseases, Tokyo, Japan. IBV-specific PCR was performed using an IBV Matrix protein gene-specific primer set (FluB-MF439: 5′-CTCTGTGCTTTGTGCGAGAAAC-3′ and FluB-MR: 5′-CCTTCYCCATTCTTTTGACTTGC-3′) [13].

Immunohistochemical analysis

Immunohistochemical staining was performed using the avidin–biotin-labelled enzyme complex method (Vectastain Elite ABC mouse IgG kit; Vector, Burlingame, CA). A monoclonal antibody (diluted 1:500 in phosphate-buffered saline) against the IAV nucleoprotein (C43) was reacted with cells/tissues at room temperature for 30 minutes [14,15]. Normal mouse IgG (Santa Cruz Biotechnology, Dallas, TX) was used as a control, and 3,3′-diaminobenzidine (DAB Substrate Kit; Vector) was used as a peroxidase substrate. Cells/tissues were counterstained with haematoxylin (Mayer haematoxylin solution; Wako, Osaka, Japan).

Clinical study

A prospective observational study designed to detect virus in faecal samples from IAV/IBV-infected individuals was conducted. The study enrolled individuals diagnosed as positive for IAV/IBV by the rapid antigen detection test (Quick Chaser Flu A,B; MIZUHO MEDY, Saga, Japan) at the Department of General Medicine of Kyoto Prefectural University of Medicine Hospital or at the Department of General Medicine of Reimeikai Kitade Hospital between December 2014 and March 2015 and who were able to provide faecal and sputum (or a pharyngeal swab) samples at that time. Minors (<20 years old) and patients with a history of serious disease (i.e. heart failure, kidney failure, cerebrovascular disorder, advanced cancer and immunodeficiency disorders) were excluded. Informed consent was obtained at the time of examination, and 22 patients (19 IAV positive and three IBV positive) were included in the analysis.

Sputum and faecal samples were tested for virus RNA. The 19 IAV-positive patients were divided into faecal IAV-positive and -negative groups for statistical analyses. Primary factors analysed were age, sex, body temperature at the time of medical examination, symptoms (general symptoms, upper respiratory tract symptoms and abdominal symptoms) and virus copy number in sputum specimens. The study was reviewed and approved by the institutional review board of the Kyoto Prefectural University of Medicine (ERB-C-273).

Clinical research (patients 1 and 2 and supplemental patient 1)

One follow-up case (patient 1) was selected from the group of patients with IAV-positive faecal samples. The patient was a 33-year-old man with no preexisting medical conditions who received symptomatic treatment only and was not treated with a neuraminidase inhibitor. The other follow-up case (patient 2) was selected from the group of patients with IBV-positive faecal samples. The patient was a 52-year-old woman with no preexisting medical conditions who received symptomatic treatment only and was not treated with a neuraminidase inhibitor.

The observation period was 35 days, and sputum and faecal samples were collected 11 times (days 1, 2, 3, 4, 5, 7, 10, 14, 17, 24 and 35). The collected sputum and faecal samples were subjected to virus isolation culture, and virus RNA was measured.

A 6-month-old male infant whose sputum and faeces tested positive for IAV (supplemental patient 1) was also included. The infant had no preexisting medical conditions, underwent symptomatic treatment only and was not treated with a neuraminidase inhibitor. The observation period was 35 days, and virus RNA was measured in all collected sputum and faecal samples.

Clinical research (patient 3)

A female patient (aged 67 years) with influenza A (diagnosed by the influenza rapid antigen detection test) received conservative treatment; however, she then developed haemorrhagic colitis and underwent colonoscopy. The patient had no preexisting medical conditions and received symptomatic treatment only (no neuraminidase inhibitor). From day 6 of illness, the patient had a temperature of 38°C or higher; low-grade fever and upper respiratory tract symptoms persisted thereafter. Abdominal pain and loss of appetite were noted on day 15 of illness and gradually worsened thereafter. Bloody stools were noted on day 22. The patient visited a