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Severe acute respiratory syndrome—a global concern—influenza virus isolated from suspected cases in Brazil from April to June 2003

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Abstract. Severe acute respiratory syndrome (SARS) is a condition associated with substantial morbidity and mortality. Coronavirus has been associated with this severe emerging disease, with a pattern suggesting droplet or contact transmission. From April to June 2003, Institute Adolfo Lutz received 16 respiratory secretions from hospitalized patients with recent history of travel to an area with local transmission of SARS. Rapid antigen detection for influenza A and B, parainfluenza types 1, 2 and 3, respiratory syncytial virus and adenovirus; electron microscopy, polymerase chain reaction (PCR), reverse transcription PCR (RT–PCR) and serologic assay were performed. Virus isolation attempts were performed in Hep-2, Vero, MDCK, NCI-H292, MRC-5, LLC-MK2 and FRhK-4. Influenza virus of type A/Panama/2007/99 (H3N2) and A/New Caledonia/20/99 (H1N2) were identified. © 2004 Published by Elsevier B.V.

Keywords: RT–PCR; Electron microscopy; Influenza; Metapneumovirus; Coronavirus

1. Introduction

A global concern at the beginning of the 21st century arises due to the emerging infectious disease in South East Asia, Guangdong Province, China. Coronavirus was identified as the etiologic agent of the highly contagious disease presenting significant

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morbidity and mortality [1]. Designated as Severe Acute Respiratory Syndrome (SARS-Co), the new emerging disease dissemination was also detected in North America, Europe and Canada, and remains confined to some areas. Brazilian Public Health Authorities established a rigorous surveillance schedule in order to monitor imported cases among international travelers arriving from areas with community transmission. The Respiratory Virus Laboratory of the Adolfo Lutz Institute received 16 respiratory samples from patients arriving in São Paulo from Japan, Hong Kong, Pequiem, Taiwan, Europe and Canada with febrile respiratory illness.

2. Material and methods

2.1. Respiratory samples

From April to June 2003, we received respiratory secretions from 16 hospitalized patients with recent history of travel to an area with local transmission of SARS.

Laboratory testing focused foremost on known respiratory pathogens. A combination of traditional methods was applied, including indirect immunofluorescence (IF), virus isolation in cell culture, electron microscopy and serology. The molecular techniques of polymerase chain reaction (PCR) and reverse transcription PCR (RT–PCR) were used to testing for the following agents: human metapneumovirus and human coronavirus.

2.2. RT–PCR

RNA extracts were prepared from 100 μl of cell culture supernatant with the NucliSens (bioMérieux) or TRIZOL (LifeTechnologies) extraction reagents. Oligonucleotide set primers used for amplification were IN-2 (+) GGG TTG GGA CTA TCC TAA GTG TGA and IN-4 (−) TAA CAC ACA ACI CCA TCA TCA, designed to conserve regions of the genus *Coronavirus*, the SARS-specific primers Cor-p-F2 (+) CTA ACA TGC TTA GGA TAA TGG, Cor-p-F3 (+) GCC TCT CTT GTT CTT GCT CGC and Cor-p-RI (−) CAG GTA AGC GTA AAA CTC ATC [1], BNIoutS2 ATG AAT TAC CAA GTC AAT GGT TAC (+), BNIinS GAA GCT ATT CGT CAC GTT CG (+), BNIinAs CTG TAG AAA ATC CTA GCT GGA G (−) and BNIoutAs CAT AAC CAG TCG GTA CAG CTA C (−) [2]. Primers for specific amplification of human metapneumovirus were also used, MPn-1 5′CTT TGG ACT TAA TGA CAG ATG3′ and MPn-2 5′GTC TTC CTG TGC TAA CTT TG3′ [3]. One-step amplification reactions were performed with the following amplification mix: 2× RT–PCR master mix, nuclease-free water, 2,5 pM/μl primers, 5 U/μl AMV RT and 5 μl RNA extract (50 μl total volume). Thermal cycling comprised 48 °C for 45 min, 94 °C for 2 min, 40 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and 72 °C for 7 min. Gel electrophoresis and ethidium bromide staining analyzed the products.

2.3. Electron microscopy

Direct electron microscopy (DEM) was performed using respiratory secretions by negative staining technique using 2% sodium phosphotungstate pH 6.4 and exanimate in a Philips 400-T electron microscope working at 80 kV [4–6].

2.4. Indirect immunofluorescence

2.4.1. Processing throat swabs and nasopharyngeal secretions

Rapid diagnosis, by using immunofluorescence assay (IF), was performed as described previously [7]. Monoclonal antibodies from the respiratory Panel 1 Viral Screening and Identification Kit (influenza A and B, respiratory syncytial virus, adenovirus and para-influenza viruses types 1, 2 and 3) from Chemicon International (Temecula, CA) were used as reagents for IF.

2.5. Processing samples for virus isolation

Nasopharyngeal secretions were vigorously shacked in a vortex mixer and 1000 U/ml of penicillin and 1000 μg/ml of streptomycin were added. Cell cultures of Hep-2, Vero, MDCK, NCI-H292, LLC-MK2, FRhK-4 and MRC-5 were inoculated. Influenza viruses isolated from cell culture were identified by immunofluorescence with monoclonal antibodies of the Respiratory Panel 1 Viral Screening and Identification Kit, (Chemicon Internantional). Following the subtype characterization by the hemagglutination inhibition test, antiserum provided by the World Health Organization (WHO) was used [2]. Isolated viruses were sent to the Influenza Branch at the Centers for Disease Control and Prevention—Atlanta, GA—for further antigenic and genetic analysis.

3. Results

Of the 16 nasopharyngeal specimens, four showed cytopathic effect in only one cell line, MDCK. Isolated viruses were identified as influenza of type A by immunofluorescence with monoclonal antibodies of the Respiratory Panel 1 Viral Screening and Identification Kit, (Chemicon Internantional). Following the subtype characterization by the hemagglutination inhibition test, antiserum provided by the World Health Organization (WHO) was used. Isolated viruses were sent to the Influenza Branch at the Centers for Disease Control and Prevention—Atlanta, GA—for further antigenic and genetic analysis. Influenza viruses were isolated in the respiratory secretions from four patients with recent travel history to Hong Kong, Pequiem, Europe and Taiwan, respectively. Three isolated viruses were characterized as A/Panama/2007/99 (H3N2) and one as A/New Caledonia/20/99 (H1N2). Among them, a mixed infection with influenza virus of type B from a 71-year-old woman from Taiwan was observed. In addition, further studies are currently being completed to help characterize a virus isolated from Canada of a suspected case and a microorganism distinguished from a virus, which has been isolated from four suspected cases of SARS.

4. Discussion and conclusion

A new emerging respiratory disease has again challenged the worldwide scientific community. The mysterious etiologic agent of unknown origin was revealed by electron microscopy as coronavirus [1].

Nowadays, in addition to the potential risk of an influenza virus pandemic, the introduction of the SARS-Co into the human population concerned the Global Public Health Authorities greatly.

This investigation also emphasizes how important it is to perform the differential diagnosis, along with SARS, when evaluating patients with febrile respiratory illness [8]. Pandemic influenza strain mechanisms are already known by the scientific community. On the other hand, regarding SARS-Co, an open field towards elucidating its origin still needs to be investigated.

Facing this new global threat, a lot of questions remain to be answered: (a) which factors have been limiting its dissemination; (b) how molecular changes have contributed to its replication in cell cultures like Vero, NCI-H292 and FRhK-4 because the human coronavirus is related as fastidious, preferring selected cell lines, organ culture or suckling mice for propagation; and (c) do the successes of the mass culling of poultry strategy adopted by the Asian Public Health Authorities, in preventing H5N1 dissemination, contribute to the closest contact between an unusual avian coronavirus host and the human species?

Phylogenetic analysis does not support recombination events in generating this new coronavirus. In addition, the discovery of the 29 extra nucleotide sequences in the closest suspected host infected with a coronavirus identical to the one that causes SARS-Co brought out the importance of follow-up of the SARS host investigation [9].

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