Infection, Genetics and Evolution 30 (2015) 55–58

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

A new member of the *Pteropine Orthoreovirus* species isolated from fruit bats imported to Italy

Alessio Lorusso *, Liana Teodori, Alessandra Leone, Maurilia Marcacci, Iolanda Mangone, Massimiliano Orsini, Andrea Capobianco-Dondona, Cesare Camma', Federica Monaco, Giovanni Savini

Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise, IZSAM, Teramo, Italy

ARTICLE INFO

Article history:
Received 5 September 2014
Received in revised form 14 November 2014
Accepted 3 December 2014
Available online 9 December 2014

Keywords:
Pteropine Orthoreovirus
Fruit bats
Italy

ABSTRACT

A novel member of the *Pteropine Orthoreovirus* species has been isolated and sequenced for the whole genome from flying foxes (*Pteropus vampyrus*) imported to Italy from Indonesia. The new isolate named Indonesia/2010 is genetically similar to Melaka virus which has been the first virus of this species to be shown to be responsible for human respiratory disease. Our findings highlight the importance of flying foxes as vectors of potentially zoonotic viruses and the biological hazard that lies in the import of animals from geographical areas that are ecologically diverse from Europe.

Fruit bats or flying foxes of the genus *Pteropus* live in the tropics and subtropics of Asia (including the Indian subcontinent), Australia, islands off East Africa and some remote oceanic islands in both the Indian and Pacific Oceans. They are known to be reservoir of many zoonotic viruses (Kuzmin et al., 2011). In addition to their role as natural reservoirs of Hendra and Nipah viruses (reviewed in Calisher et al., 2006), they have also been described as a reservoir for Orthoreoviruses. These viruses are members of the *Reoviridae* family and can infect mammals including humans. From fruit bats fusogenic Orthoreoviruses were first isolated in Australia in 1968, the virus was called Nelson bay virus (Gard and Marshall, 1973). The zoonotic potential of the Orthoreoviruses from bats was first evidenced in 2006 when a man suffered from fever and respiratory symptoms after exposure to a flying fox. The human isolate was called Melaka virus (Chua et al., 2007), a strain strictly related to Nelson bay virus. Afterward, other viral isolates genetically and antigenically related to the prototype Nelson Bay virus such as Kampar, Miyazaki-Bali/2007, HK23629/07 and Sikamat have been isolated from patients with respiratory tract infections (RTI) (Chua et al., 2008; Yamanaka et al., 2014; Cheng et al., 2009; Chua et al., 2011). All these viruses have been shown to have limited human-to-human transmission. Together with Pulau and Xi river viruses, isolated from flying foxes in 1999 in Malaysia and in 2010 in China, respectively, (Du et al., 2010; Pritchard et al., 2006) they form the recently proposed *Pteropine Orthoreovirus* species (Table 1). The present study describes a new strain called Indonesia/2010 isolated from a salivary swab taken from one flying fox imported to Italy from Indonesia.

Salivary and fecal samples of thirty flying foxes (*Pteropus vampyrus*) imported from Indonesia were collected under general anesthesia and tested at the laboratories of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise (IZSAM) for the presence of Hendra and Nipah viruses by nucleic acid recognition methods (OIE World Organization for Animal Health, Terrestrial Manual-2013). The animals were straightly transported under sanitary conditions from the quarantine station of the Fiumicino airport (Rome) to a quarantine center in the municipality of Gatteo (Emilia Romagna Region). They remained in quarantine for 30 days before being shipped to an animal facility in Northern Europe. Samples were also suspended in PBS with antibiotics (C. penicillin 100,000 I.U./ml and streptomycin 100 μg/ml) overnight at 4 °C and inoculated in duplicate onto freshly confluent monolayers of *Aedes albopictus* clone C6/36 cells, *Culicoides sonorensis*-derived KC cells, Madin Darby canine kidney (MDCK) cells and African green monkey kidney (Vero) cells in 6 well tissue culture plates. Plates were then incubated at 37 °C or 28 °C in 5% CO_2 and monitored daily for the presence of cytopathic effects (CPE). In case of visible syncytial cytopathic effects the cell culture medium was collected and stored for further analysis. Extraction and purification of total RNA was performed from Vero cells infected with passage 2 of Indonesia/2010 isolated from the salivary inoculum. dsRNA was purified (Lorusso et al., 2014) and separated on 1% agarose gel. Vero confluent cell monolayers in 24-well cluster plates

* Corresponding author. Tel.: +39 0861332440; fax: +39 0861332251.
E-mail address: a.lorusso@izs.it (A. Lorusso).

http://dx.doi.org/10.1016/j.meegid.2014.12.006
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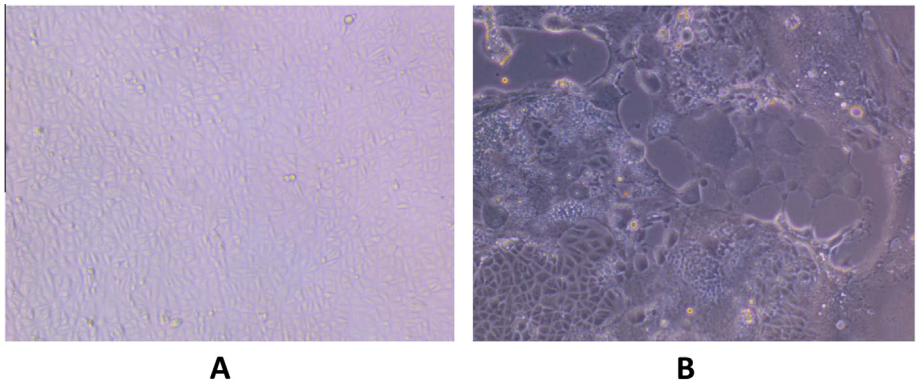

Fig. 1. (A) and (B) Cell-culture characteristics of Indonesia/2010 on Vero cells. (A), mock inoculated; (B), CPE 48 h post infection.

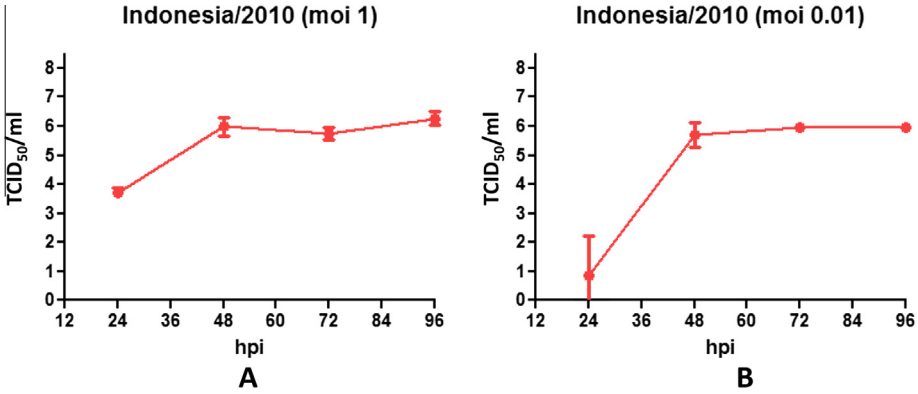

Fig. 2. (A) and (B) Growth of Indonesia/2010 *in vitro*. Confluent monolayers of Vero cells were infected in triplicate with Indonesia/2010 at a multiplicity of infection (moi) of 1 (A) and 0.01 (B) for 1 h in minimal medium, after which the monolayers were washed three times. Samples were collected at 8, 24, 48, 72 and 96 h post infection, and virus titers were determined in duplicate by $TCID_{50}$ on Vero cells. The mean of the log-transformed titers are shown ± the standard error of the mean (SEM).

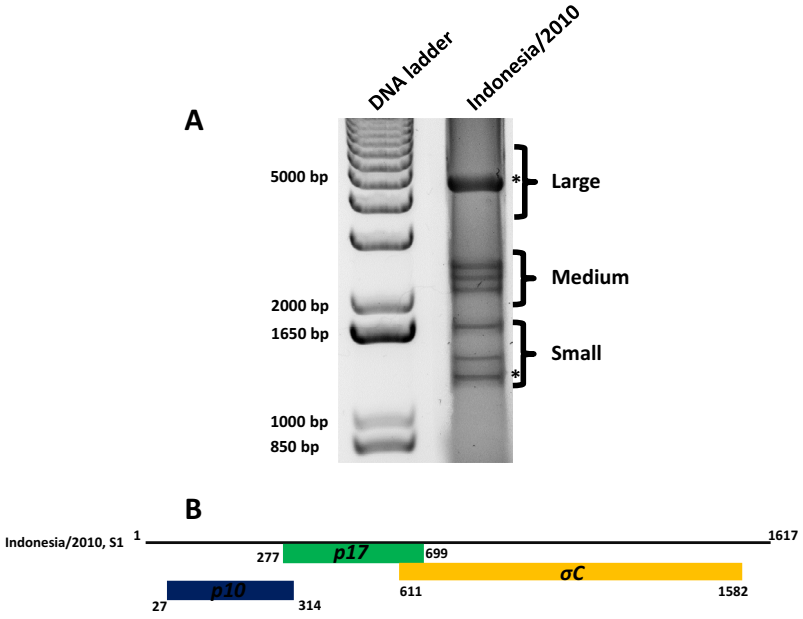

Fig. 3. (A) and (B) Genome segments of Indonesia/2010 separated on 1% agarose gel. Asterisks indicate the presence of comigrating bands where more than one segment is present. The classes of genome segments (L, large; M, medium; S, small) are labeled on the right (A). ORFs organization of the tricistronic S1 segment. The line on the top represents the RNA genome and the shaded boxes underneath show protein coding regions in reading frames (top to bottom). Numbers refer to size, in base pairs, of S1 and to the first and last nucleotides of the individual ORFs including the stop codons. The names of the putative encoded proteins are indicated within the shaded boxes (B).

Table 1
Pteropine Orthoreoviruses isolated since 1968 so far.

Virus name	Year	Host	Location
Nelson Bay	1968	Bat	Australia
Pulau	1999	Bat	Malaysia
Melaka	2006	Human	Malaysia
Kampar	2006	Human	Malaysia
HK23629/07	2007	Human	Hong Kong
Miyazaki-Bali/2007	2007	Human	Indonesia/Japan
Sikamat	2010	Human	Malaysia
Xi River	2010	Bat	China
Indonesia/2010	2010	Bat	Indonesia/Italy

Table 2
Highest% nucleotide identity for each gene segment of Indonesia/2010 as compared with existing Pteropine Orthoreoviruses.

Indonesia/2010	% ID	Reference	Ref. acc. Nos.	Coverage (%)
L1	96%	Pulau	JF342666	98
L2	95%	Pulau	JF342667	85
L3	86%	Pulau	JF342668	92
M1	86%	Pulau	JF342669	88
M2	92%	Pulau	JF342670	90
M3	92%	Melaka	JF342665	94
S1	77%	Nelson bay	AF218360	100
S2	92%	Pulau	AY357731	96
S3	91%	Nelson bay	AF059726	90
S4	96%	Melaka	EF026046	90

were also infected with the second passage on Vero cells of Indonesia/2010 in order to obtain the MOI (multiplicity of infection) of 1 and 0.01. Supernatants were collected at 8, 24, 48, 72 and 96 h post infection. The virus was tested in triplicate in three independent experiments. Titres at each time point were calculated by $TCID_{50}$ on Vero cells (Reed and Muench, 1938). For Next Generation Sequencing, samples were processed according to protocols recently developed at the IZSAM (Marcacci et al., 2014; Lorusso et al., 2014). Sequencing was performed by the PGM Ion Torrent Platform. After quality trimming, reads were *de novo* assembled using MIRA (version 4.0rc4). Ambiguous nucleotides were solved by local reads remapping by BWA-MEM followed by alignment visual inspection. Phylogenetic analysis was conducted using the MEGA6 software package with the Maximum Likelihood (ML) algorithm. To assess the robustness of individual nodes on the phylogenetic tree, a bootstrap resampling analysis (100 replications) using the neighbor-joining method has been performed.

All salivary and fecal swabs were negative when tested for Hendra and Nipah viruses and therefore they were propagated in cell culture. Syncytial CPE (Fig. 1) was evidenced in Vero cells inoculated with salivary and fecal inoculums of one single individual out of the thirty sampled animals 48 h after incubation at 37 °C. Unfortunately we did not have any detailed information regarding the precise location where the animals were captured. Therefore, the isolate was named "Indonesia/2010" according to the country of origin of the flying foxes unlike the other members of the

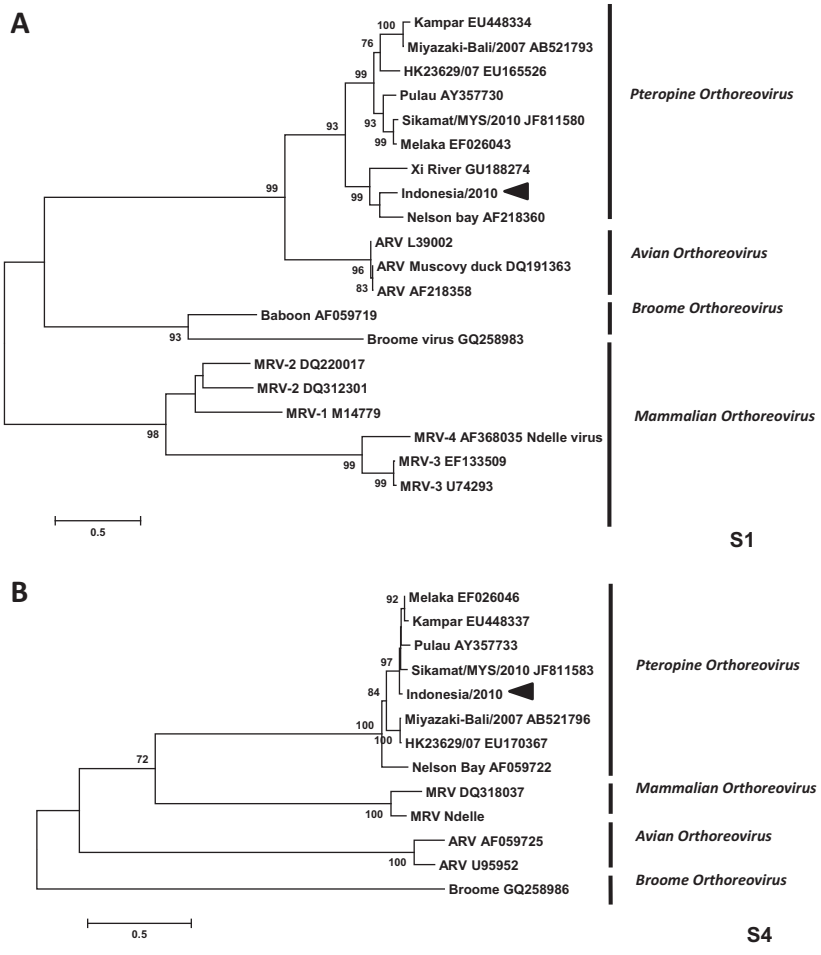

Fig. 4. (A) and (B) Maximum-Likelihood trees inferred from multiple nucleotide sequence alignment of (A), S1 and (B) S4. S1 and S4 sequences of Orthoreoviruses of multiple isolates of *Pteropine Orthoreovirus*, *Mammalian Orthoreovirus (MRV)*, *Avian Orthoreovirus (ARV)*, *Broome* (from bats) viral species from different geographical areas available on Genbank, were recruited. Bars indicate the estimated numbers of nucleotide substitutions per site. Bootstrap values ⩾70 are indicated. Reptilian Orthoreoviruses have not been included in the analysis.

Pteropine Orthoreovirus species whose names refer to municipalities. Indonesia/2010 was capable of growing and causing CPE also in MDCK, C6/36 but not on KC cells. KC is a cell line routinely used for the first isolation attempt of Bluetongue virus (BTV), genus *Orbivirus*, family *Reoviridae*. Growth curves are depicted in Fig. 2A and B. Indonesia/2010 was shown to have a genome constellation of 10 segments. All gene segments were amplified for most of their length except in some cases for the 5′ and 3′ ends. The highly conserved genome terminal sequences at the 5′ end of the positive-sense RNA was GCUUUA for gene segments 1, 2, 3, 4 and 7; GCUUAU for gene segment 5 and 10; GCUCAA for gene segment 9. Indonesia/2010 was shown to have a genome organization similar to Nelson Bay, Pulau, Melaka and Kampar viruses of the *Pteropine Orthoreovirus* species. Separation of dsRNA on 1% agarose gel was similar to that of Melaka (Chua et al., 2007) and Pulau viruses (Fig. 3A). For each gene segment, the highest nucleotide% identity, as compared with existing *Pteropine Orthoreovirus* species is listed in Table 2. Sequences have been deposited in GenBank under the accession numbers (KM279380–KM279389). Taxonomically, Orthoreoviruses are commonly grouped on the basis of the S1 segment (Song et al., 2008; Dermody et al., 1990). Based on sequence data, S1 gene segment of Indonesia/2010 was shown to be tricistronic (Fig. 3B). Phylogenetic trees for gene segments S1 and S4 are depicted in Fig. 4A and B. S1 and S4 analyses clearly indicate that Indonesia/2010 is a member of the *Pteropine Orthoreovirus* species, distinct from Broome, Avian and Mammalian Orthoreoviruses. Phylogenetic analysis of the remaining gene segment sequences confirms the clustering of Indonesia/2010 into the *Pteropine Orthoreovirus* species (data not shown).

To the best of our knowledge, Indonesia/2010 is the ninth member of this viral species, the fourth isolated from flying foxes. Indonesia/2010 has been isolated from healthy and legally imported flying foxes. Numerous new viruses of bat origin emerged in the recent decades (Kuzmin et al., 2011; Calisher et al., 2006). Some of them might have a significant impact on human and animal health, tourism, and trade. Even if the zoonotic potential of Indonesia/2010 is still unknown, it cannot be excluded considering the patho-biological features of the other members of this viral species. The segmented nature of its genome poses additional risks regarding the potential onset of novel reassortant viruses with unpredictable biological properties. In Europe, for instance, bat borne non fusogenic Mammalian Orthoreoviruses species have been recently described in vespertilionid bats in Germany and Italy (Kohl et al., 2012; Lelli et al., 2013). Although the advance of the diagnostic technology has certainly improved the workflow to identify new viruses, it is commonly accepted that environmental changes which result in greater interaction of human and wildlife animals, are mainly responsible for the increased spillover events observed in the recent years. Pteropine Orthoreoviruses are able to cause RTIs in humans, therefore physicians should consider these pathogens in the diagnostic workflow particularly with patients coming from areas where flying foxes live or with patients who had contacts with them. Overall, this study highlights once more the importance of flying foxes as vectors of potentially zoonotic viruses and the possible biological hazard that lies in the import of animals from geographical areas that are ecologically diverse from Europe.

Acknowledgments

Funding was provided by the Italian Ministry of Health. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the IZSAM.

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