SHORT COMMUNICATION

Development of a serum neutralization assay to detect Pteropine Orthoreovirus Indonesia/2010 neutralizing antibodies

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Summary
Pteropine Orthoreoviruses (PRVs) are fusogenic bat-borne orthoreoviruses that cause flu-like upper respiratory tract infections in humans. The presence of this group of viruses in bats and humans has been well documented in areas where their biological reservoirs – fruit bats (family *Pteropodidae*) – live densely. In the present study, a serum neutralization (SN) assay to detect neutralizing antibodies against PRV Indonesia/2010 isolate was set up and used to assess the seroprevalence of this virus in Italian domestic animals. The new developed assay was able of detecting PRV neutralizing antibodies in the hyper-immune polyclonal serum produced in rabbits (titer of 1:160). The negative serum was negative at all tested dilutions. No cross-reactions have been evidenced neither against reference MRVs nor against their respective hyper-immune sera. Eight hundred and fifty-three serum samples collected from 524 bovines, 271 small ruminants, and 58 horses (all used as sentinel animals in the Bluetongue and West Nile disease National surveillance program) were also tested with the new developed SN assay. According to the results of this survey, neither PRV nor PRV cross-reacting viruses antibodies have been demonstrated in Italian domestic animals. However, the new developed SN assay could be a very valuable diagnostic tool to detect infection in animals and humans.

Test di siero-neutralizzazione per rilevare e quantificare anticorpi nei confronti del ceppo Indonesia/2010 del Pteropine Orthoreovirus

Parole chiave
Pteropine Orthoreovirus,
Siero-neutralizzazione,
Sieri,
Animali domestici.

Riassunto
I Pteropine Orthoreovirus (PRVs) sono ortovirus fusogenici trasmessi dai pipistrelli che causano negli esseri umani infezioni simil-influenzali del tratto respiratorio superiore. La presenza di questo gruppo di virus nei pipistrelli e negli esseri umani è stata ben documentata in aree dove i pipistrelli (*Pteropodidae*), loro ospiti biologici, vivono numerosi. In questo studio abbiamo valutato un test di siero-neutralizzazione per il ceppo PRV Indonesia/2010 e testato, per la presenza di anticorpi neutralizzanti (Abs), 844 campioni di siero di animali domestici utilizzati come sentinella nei piani di sorveglianza nazionali per Bluetongue e West Nile. Tutti i campioni testati sono risultati negativi. Nel complesso, abbiamo sviluppato un test per la diagnosi sierologica dell'infezione da PRV che può essere utilizzato per analizzare campioni di siero umano o animale.

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According to the International Committee on Taxonomy of viruses (ICTV 2014), the family *Reoviridae* is composed by 2 subfamilies: *Sedereovirinae* and *Spinareovirinae*. *Spinareovirinae* includes 9 genera, *Orthoreovirus* genus is one of these. This genus is composed by 5 species including Mammalian Orthoreovirus, Avian Orthoreovirus, Baboon Orthoreovirus, Reptilian Orthoreovirus, and Nelson Bay virus, also known as Pteropine Orthoreovirus. The first detection of a Pteropine Orthoreovirus (PRV) viral species dates back in 1968 in Australia from fruit bats, called flying foxes (family *Pteropodidae*) (Gard *et al.* 1973). The prototype virus was called Nelson Bay virus. The zoonotic potential of PRVs has been ascertained in 2006, when the case of a man exhibiting fever and respiratory flu-like symptoms after exposure to a flying fox was reported (Chua *et al.* 2007). The human isolate was called Melaka virus, a strain strictly related to Nelson bay virus (Chua *et al.* 2007). Other viral isolates genetically and antigenically related to the prototype Nelson Bay virus have been isolated from patients with respiratory tract infections, such as Kampar, Miyazaki-Bali/2007, HK23629/07, HK46886/09, HK50842/10 and Sikamat (Chua *et al.* 2008, Cheng *et al.* 2009, Chua *et al.* 2011, Yamanaka *et al.* 2011, Wong *et al.* 2012). Together with Pulau and Xi river viruses isolated from flying foxes in 1999 in Malaysia and in 2010 in China (Pritchard *et al.* 2006, Du *et al.* 2010), respectively, these viruses form the recently proposed Pteropine Orthoreovirus species.

Recently, a novel member (Indonesia/2010) of this viral species has been identified in fruit bats legally imported to Italy from Indonesia (Lorusso *et al.* 2015). At the same time, a new isolate named Cangyuan virus has been detected in the intestinal content of a fruit bat (*Rousettus leschenaultia*) residing in China's Yunnan province (Hu *et al.* 2014). The presence of these viruses has been reported also in the Philippines (Taniguchi *et al.* 2017). While the zoonotic potential has been proved for Pteropine Orthoreovirus, to date, no information is available on the capability of these fusogenic orthoreoviruses to infect domestic animals and on whether they have circulated or are circulating in Italy. Therefore, we developed a specific serum neutralization assay for detecting neutralizing antibodies against Indonesia/2010 strain and used it to test several serum samples collected from Italian domestic animals. This test could be a valuable tool for detecting infection in tourists coming back from endemic areas.

To set up the SN assay, a positive control serum was produced in rabbits. Briefly, after passaging twice onto African green monkey (Vero) cells and inactivating, Indonesia/2010 strain was injected in rabbits according to the method described by Ronchi and colleagues (Ronchi *et al.* 2012). Sterile cell culture medium was also given to rabbits to obtain negative control serum. The specificity of the positive serum was assessed against the Mammalian Orthoreoviruses (MRV-1 Lang, MRV-2 Jones and MRV-3 Abney viruses), which are known to circulate in Italy among both animals and humans (Lelli *et al.* 2013). Serum samples to be tested were inactivated at 56°C for 30 minutes. Starting from a dilution titer of 1:2, serial 2-fold dilutions were made in 96 well flat bottom microtiter plates. Indonesia/2010 strain at a titer of 100 $TCID_{50}$ (50% tissue culture infecting dose) was added to each dilution. Thereafter, the mixtures were incubated at 37°C for 1 hour, and 10^5 Vero cells were added to each well. The plates were incubated at 37°C for 5 days. Starting from the third day after incubation, the plates were checked for cytopathic effect, and the antibody titer was defined as the reciprocal of the highest dilution of the serum that showed 100% neutralization. Positive and negative control sera were included in each plate. To assess the sensitivity of the assay, dilutions of the positive serum samples were blindly used. Furthermore, to investigate the presence of serological cross-reactions with MRVs, hyper-immune sera produced for MRV-1 Lang, MRV-2 Jones and MRV-3 Abney isolates were included in the test. Once set up, the assay was also used to test serum samples of various domestic animals. Eight hundred and fifty-three serum samples from different domestic animals from Italy (524 bovines, 271 small ruminants, and 58 horses) were collected in 2015 within the activities of the National Bluetongue and West Nile disease surveillance plans (Giovannini *et al.* 2004a, Giovannini *et al.* 2004b; Ministry of Health 2014, DGSAF 2015). The selected animals were those used as sentinels in these programs and originated from all Italian regions. Dilutions of the 853 serum samples were tested in duplicate as previously described, and prevalence (*P*) and 95% confidence interval (CI) were assessed.

The new developed assay was able of detecting PRV neutralizing antibodies in the hyperimmune polyclonal serum produced in rabbits (titer of 1:160). Negative serum was negative. No cross-reactions have been evidenced neither against reference MRVs nor against the respective hyper-immune sera. All tested field serum samples turned serologically negative for PRVs (95%CI: 0-0.35%).

Fusogenic bat-borne orthoreoviruses of the Pteropine Orthereovirus genus have been described as one of the emerging causes of upper-respiratory tract infections in humans within the areas where Pteropid bats live (Voon *et al.* 2015). To date, there is no record of PRVs circulating in Europe. In the present study, we first develop a serum-neutralization assay for Indonesia/2010 strain and then assess its prevalence in domestic animal sera. According

to the results of this survey, neither PRV nor PRV cross-reacting viruses antibodies have been demonstrated in Italian domestic animals. Since we don't know if this virus is capable of infecting domestic animals, it is difficult to establish whether this finding depended on the inability of this virus to infect domestic animals or on the fact that it has not circulated or is not circulating in Italy. Although, MRVs, which belong to a species closely related to PRVs, have been shown to circulate in Italian and European bats (Lelli 2013a, Khol 2012), the lack of evidence of PRV circulation in Italy may also be due to the fact that the species of bats involved in their transmission cycle do not naturally live in Europe. A proper surveillance on human sera would have been more beneficial as well as a serological survey in domestic and wild animals in geographical areas with a high prevalence of fruit bats, which have been shown to be the biological reservoir of PRVs. Indeed, human PRV acquired infections have also been linked to tourism in these areas (Wong *et al.* 2012). Even if the specificity of our assay was not tested with other representative PRV isolate antisera, considering that PRV did not cross react with representative MRVs, it seems that this new developed SN assay is quite specific and therefore it could be a very valuable diagnostic tool to detect infection in tourists coming back from endemic areas.

In conclusion, we developed a test for serological diagnosis of PRV infection which can be used to screen both animal and human serum samples. Physicians should consider PRV infection in the diagnostic workflow of patients coming from areas where flying foxes live and of patients who had contacts with them.

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