The use of convalescent sera in nsIEM for the detection of not suspected and/or new viral agents of veterinary relevance

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Nowadays, for direct virologic diagnosis, the most frequently used methods are ELISA and PCR. Indeed, negative staining electron microscopy (nsEM) may be still used when viruses unknown or that can not be isolated *in vitro* are implicated as caused of infection. Among EM methods, immuno-electronmicroscopy (IEM) is the more sensitive and specific. In fact, IEM permits to examine directly in EM and identify viruses in clinical specimens adding a second dimension, antigen specificity, to the primary morphological identification. It thus makes possible 1) to differentiate viruses belonging to the same family, to serotype a viral agent; 2) to simplify the identification of virions which are pleomorphic i.e. do not present peculiar morphology (elusive viruses), present in low concentration (10^2-10^3 enrichment), present in "dirty" samples (complex size); 3) to improve knowledge on the viral structure and in particular the specific binding sites and the subviral components.

The aim of this study is to support, through the presentation of practical cases, the value of the application of convalescent sera in IEM for the detection and identification of viral particles in samples of animal origin.

"Convalescent sera" are defined as those sera obtained from blood samples taken from animals of the same group that suffered from a clinical outbreak of disease, associated or not with mortality, 15-20 days after the peak of the outbreak. Usually more blood samples (2-20 according to the animal species and the size of the herd/group) are used and pooled. The blood samples are firstly put at 37°C for 1-2 hours and then overnight at 4°C. After removal of the clot they are centrifuged at 300g for 20 minutes, filtered through a 0.45µm Millipore filter, inactivated at 57°C in water bath for 30 minutes, centrifuged at 10000g for 30 minutes and filtered again through a 0.22 µm Millipore filter. Before the use **e**ach serum, or pool of sera, is examined by EM to assure that it does not contain viral particles and lipid and protein debris. The sample is then tested using serial dilutions of the serum to ascertain its optimal titre giving a clear immunoaggregation of viral particles. Initially, 1:2, 1:20, 1:200 and 1:1000 dilutions are tested, then, if necessary according to results, intermediate dilutions (1:10, 1:50, 1:100, 1:500 etc.) are used. The optimal dilution is the one that 1) gives immuno-aggregates of an adequate size; 2) the halo of antibodies around virions is not so thick; 3) the morphology of the virion is not altered. The applied controls to exclude the spontaneous aggregations of virions include: 1) sample with no serum; 2) incubation with a pre-serum (if available); 3) incubation with another serum.

Negative staining IEM of viral suspensions obtained form different type of samples (organ homogenates, faecal extracts, urine, blood, skin scrapings etc) is easy and quick to perform (around 4 hours from arrival of the sample to observation) and offers quite good level of sensitivity, being the threshold level of IEM around 10^3-10^4 particles/ml. In our laboratory the IEM method applied takes advantage from the use of Airfuge Beckman ultracentrifugation as enrichment step. The samples (organ homogenates 1:5 w/v or faecal materials 10% v/v in distilled water) are freezed and thawed twice, the supernatant is

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harvested and centrifuged at 4,000 g for 20 min. and at 9300 g for 10 min. for clarification. Before the ultracentrifugation, the 2nd supernatant from clarification (85 µl) is incubated for 1hr at 37°C with an equal amount of the different serial dilutions of the convalescent serum upon gently agitation. Then, such mixture is ultracentrifuged in Airfuge Beckman for 15 min. at 21 psi (82000 g). The Airfuge is fitted with an A100 rotor holding six 175 µl test tubes in which were put specific adapters for 3 mm grids which allow direct pelleting of viral particles on 200 mesh carbon-coated Formvar copper grids. The grids stained with 2% NaPt pH 6.7 are observed with a TEM Philips CM10 at 19-25000x. The identification of viral particles is achieved on the basis of the morphological characteristics typical of each viral family. Both immuno-aggregated particles and other virions not clumped by the used sera may be detected.

The examples of the use of convalescent serum to detect "new" and emerging viruses by IEM comes from the activity of the EM laboratory of our governmental Institute along a 20 years period by examining samples of different animal species as part of the diagnostic protocols of identification of pathogens of veterinary relevance. They include: 1) Porcine Epidemic Diarrhea Virus (PEDV) [6]; 2) Porcine Circovirus (PCV2) [2]; 3) Porcine Torovirus (PToV) [4]; 4) Turkey rota- and astrovirus [5]; 5) Pheasant parvovirus-like [3]; 6) Lagovirus (RHD and EBHSV) [1]. In all these cases, the use of "convalescent sera" was a key step for the identification of virions. Therefore, convalescent sera in IEM may be used in the case of clinical outbreaks when emerging o re-emerging virus could be involved as etiological agents and in view of the possible failure of alternative diagnostic methods (no immunological reagents or primers available, clinical suspect not indicative etc).

In conclusions, the undirected "open view" of conventional TEM and its "catch all" potential is further improved by the use of convalescent serum for IEM examination of clinical samples which can contain new, emerging or elusive viruses.

1. Capucci l, et al. Rev. sci. tech. Off. Int. Epiz., 10 (2) (1991) pp347-370.
2. Cerioli M. et al., Proc. 4th International Symposium on Emerging and Re-emerging Pig Diseases. Rome, 29 June-2 July 2003, p224-225.
3. Gelmetti G. et al., J. Vet. Diagn. Investig., 8 (1996) pp108-112.
4. Lavazza A. et al., Proc. 14th International Pig Veterinary Society. Bologna, Italy, 7-10 July 1996, p131.
5. Lavazza A et al., Proceedings of the 14th Congress of the "World Veterinary Poultry Association" Istanbul (Turchia) 22-26 Agosto 2005 p228.
6. Martelli P. et al., Vet. Rec. 162 (10) (2008) pp307-310.

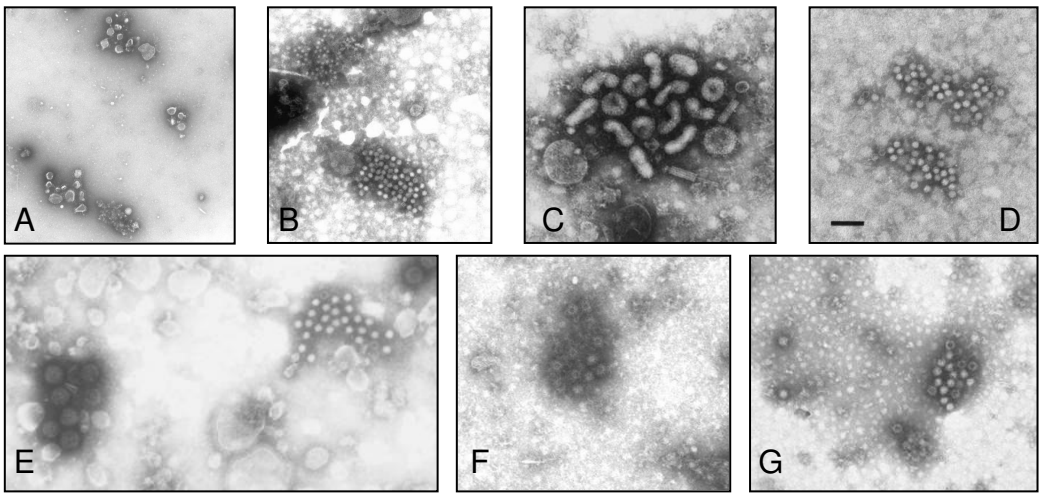

Figure 1. EM pictures of virions identified using nsIEM (NaPT 2%) Bar = 100nm A = PEDV coronavirus B = pig circovirus; C = pig torovirus; D = pheasant parvovirus; E = rotavirus associated to astrovirus; F = rabbit lagovirus (RHDV); G = hare lagovirus (EBHSV).

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