Proceedings of the 9th International Congress of World Equine Veterinary Association

Jan. 22 - 26, 2006 - Marrakech, Morocco

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CURRENT TRENDS IN EQUINE INFECTIOUS DISEASES DIAGNOSIS

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Summary

Even if the traditional methods of diagnosis remain largely employed in the field, numbers of new technologies were developed these last years, offering to the biologists and to the equine practitioners, reliable, fast and powerful tools for equine veterinary medicine. The equine diseases which have a strong economic or high-risk or epidemiological impact are logically those for which these methods experienced the greatest development, in particular for early detection of antigen or toxin (viruses, bacteria, parasites) and for spotting of fraction of genome thanks to the improvement of knowledge in molecular biology. Thus regarding influenza, or viral arteritis, strangle, herpesvirosis, leptospirosis or clostridiosis, the fast detections can be performed but also the genomic characterization of the isolated strains. These data interest also vaccines producers because they make allow thorough studies to adapt the vaccine antigens to the circulating and particularly pathogenic strains. Some examples illustrating these remarks are given through results of current international work.

Introduction

The major activity of specialized laboratories in veterinary biology and in particular in equine pathology were often based on methods of diagnosis allowing the identification of pathogens by culture (virus or bacteria) or by titration of the antibodies mainly based on techniques of seroneralisation, complement fixation, agarose gel immunodiffusion or various types of “Enzyme linked immunosorbent assay” (ELISA). The relative popularization of the methods of genome amplification (Polymerase Chain Reaction) truly improved veterinary biology and the diagnosis of equine infectious diseases by offering on one hand a very high specificity and on the other hand the possibility of countering the lack of sensitivity of the culture methods, caused by the delays and the conditions in shipping, not always easy to control in current veterinary practice (Schmitt B 2005). One will quote in example, the problems arising from the tracking of the contagious metritis and the future suggested by PCR techniques for these examinations, or the defects of sensitivity sometimes very prejudicial for the detection of the equine arteritis virus in the stallion semen and the improvement of the sanitary arrangements since the systematic recourse to genome amplification said “traditional” or in “real time”.

The genetic interest and its contribution were not limited to the tools for detection of pathogens but also in the improvement of knowledge on the physiopathology of these micro-organisms and in some of the virulence mechanisms. So the proteins could be better used, not only for their use in the serologic methods (plates sensitizing for ELISA methods with purified proteins, specific, been dependant on the virulence mechanisms…) but also in the improvement of the means of vaccination (sub unit vaccines, deleted vaccines…). One will quote for example the plasmidic factors associated with virulence of the bacterial strains of Rhodococcus equi or the development of ELISA tests for this fatal disease of the foal, based on virulence associated proteins and the early detection of the antibodies associated with the evolution of the disease. One will be able to also mention the genetic follow-up of the equine influenza strains isolated in various countries and the improvement of the commercial vaccines available on the market and adapted to these changes. The use of the genetic engineering allows also today to use synthetic peptides or recombinant antigens for the tracking of disease of lists A and B of the “international office of epizooties” (O.I.E.) such as African Horse sickness, equine infectious anemia or West Nile Virus disease.

The methods of genome amplification also modified the approach of some diseases in term of incidence in the livestock or in some countries previously presenting a safe status (West Nile, Babesiosis, Ehrlichiosis…) because they opened possibilities of detections in tissues where cultures (virus, bacteria or parasites) did not offered enough sensitivity. A number of large investigations regarding the infectious
causes of abortion in mares with this type of molecular tools showed for example, that Chlamydia, Leptospira, Coxiella, or equine herpes virus type 4, could be real causes of foetus mortality. It is for this reason necessary to take precautions when one wants to qualify a disease of “emerging” for horses, because these methods make it possible to better detect sick horses. The systematic conservation of DNA or RNA from samples for retrospective studies in the main research centers, should lead to this type of conclusion in the future. As often, the advantages of a method, available from now on in routine for the experts, should not occult essential care. Indeed, all these methods of amplification make it possible to work with less constraints in term of delays in shipping and viability of the germs to be spotted since calling upon their genetic code (ADN or ARN) but they also require work in laboratory on samples of “very small test specimens”. Less than 500µL for fluids (blood, semen, urine, bronchoalveolar liquid,...) or less than one gramme for tissue or faeces and this can represent a heavy disadvantage if the samples are little “charged” in pathogenic organisms (viral arteritis in the trophoblastic cells for example, herpesvirus in the cerebrospinal fluid) or heterogeneously distributed in the sample (blood parasites, viral abortion with localized placental lesions…)

We will hold a review, focusing on the current methods of diagnosis, of some essential diseases for the horse economy, and some others emerging or particularly monitored all over the world.

**Herpes viruses**

Nine equid herpesviruses have been identified (Patel et al. 2005) but the diagnosis of herpesvirus disease in horses cannot be made on the basis of clinical signs alone and requires laboratory assistance. Current knowledge and diagnosis applications have been performed in the last years, showing that some field strains could present very different behaviour from mare to foal or other adult. ELISA tests specific for EHV1 or EHV4 have been proposed to differentiate the circulating pathogen in stud farms even if complement fixation have a quite good cross reacting response for the two viruses. Placenta and the associated fetus organs are necessary for abortion diagnosis (Smith et al., 2004). Several PCR tests have been published and have been shown to facilitate the diagnosis in most cases. However, all these methods present the same limits. As EHV-1, all herpesviruses appear to establish lifelong latent infection. The detection of the DNA by conventional PCR does not allows the differentiation between the latent and infection statuses even if some studies report that latent carriers could be PCR positive in lymph nodes and trigeminal ganglia but negative in blood. Some results support the hypothesis that a significant difference exists between the numbers of virus observed in infection or latent state and that this difference could be demonstrated with an adapted molecular biology tool. Real time and quantitative PCR could be one of the solutions to this practical and field diagnosis. Latency can be confirmed by detection of specific latency associated transcripts (LAT) (Borchers et al., 2002). Further studies could allow differentiation between infectious and latent equine herpes virus type 1 by quantification of genomes with real time PCR methods. The next step will be validation of this new molecular tool on biological respiratory fluids.

**Strangles**

This worldwide disease has known recent diagnostic improvements thanks to the spread of serological testing based on specific ELISA detecting antibodies directed against the SeM protein of the bacterium (Sweeney et al., 2005) and making it possible to evaluate the status of the horse with respect to the disease. Serology for strangles has no value for diagnosis of clinical disease and chronic shedders. It may be helpful, however, in situations when an internal S. equi abscess is suspected and a culture cannot be obtained. It is also helpful to determine the need for vaccination as vaccination of horses with very high titers has been shown to have a greater likelihood for the development of purpura hemorrhagica. In addition, specific PCR differentiating the bacteria (Streptococcus equi subs. equi) of the other streptococcus made possible to improve the diagnosis but also tracking of the asymptomatic carriers, which are often the reservoirs of the disease in stables or stud farms (Alber et al., 2004). PCR is considered to be approximately 3 times more sensitive than culture. Commercial vaccines using attenuated and deleted strains, it is now possible to use new molecular tests, able to recognize and differentiate vaccinal or wild strains.
Contagious equine metritis

A common point with strangles and contagious metritis is notably the relative weakness of the bacteria, during transport and confronted with the ambient flora of the sample. Molecular biology could also in the future brought suitable solutions to be freed of this inconvenient. Few studies, are reporting good results demonstrating that PCR could be proposed to sanitary authorities for the diagnosis or the survey of this contagious disease (Matsuda et al., 2003; Bleumink-Pluym et al., 1994).

Leptospirosis

Leptospiral infections occur frequently and Leptospira are most often associated with cases of uveitis and more rarely with cases of abortion, stillbirth, and neonatal death (Donahue et al., 1995). Most of the studies are based on serological testing using Micro-Agglutination Testing (MAT) but rarely confirmed by culture of tissues because of the lack of sensitivity of the method with field samples. PCR has increased use for the diagnosis of infectious diseases caused by this kind of micro-organisms. Initially, PCR techniques were developed to detect isolates belonging to the Leptospira genus in clinical samples from animals or humans. Then, efforts have been made to design primers for the specific amplification of DNA sequences from pathogenic Leptospira. More recently, the hap1 gene was proposed as a PCR target for differentiation between pathogenic and saprophytic Leptospira (Branger et al., 2005; Leon et al., 2005) As mentioned in our introduction, ELISA methods are not sensitive enough to be used as official test or control tools because of the lack of knowledge concerning pathogenicity allowing specific detection of antibodies.

Rhodococcusosis

The serological method was clearly improved thanks to the knowledge obtained by some teams. The principle of these ELISA tests (Giguère et al, 2003) is mainly justified by the detection of the antibodies directed against the virulence proteins of the bacteria. This proteinic complex particularly well studied (Prescott et al., 1996; Takaï, 1997) is the major mechanism of bacterial virulence and the expression of its pathogenic capacity to infect the foal in the course of infection. Thus it seemed logical in an early routine test, to target the evaluation of the specific immune answer using this protein complex. As example, 5 virulent strains have already been identified in Normandy (Takai et al, 1999). Some works (Giguere et al. 2002) have shown that none of the serological tests currently available allow distinction between foals with clinical disease and foals on the same farm that never develop respiratory disease. At least at this point based on the information available in the literature, ELISA should not be recommended as a serological diagnosis of R. equi infections.

In theory, this kind of test should detect a response 7 or 10 days after the appearance of the first clinical signs. It is a test of confirmation for the practitioner. It is also usable at ou for the “screening” a horse population to evaluate the extent of the infection on a group of foals or to check the vaccine status of the latter. In the event of clinical suspicion, this test must always be accompanied by a blood cell count, a fibrinogen or serum amyloid A measurement. The direct research of the pathogen in culture in the respiratory tract is not always easy (Takai, 1997). The PCR became often an essential recourse because it also makes possible to check the presence of the virulence (or virulent ?) plasmid on the colonies of isolated bacteria. The large advantage of bacterial culture remains in the diagnosis of certainty and in addition the possibility to carry out an antibiogram for example. It is known today that in France, 2% (some reports in the USA suggest that up to 10% of isolates are resistant to rifampin, erythromycin or both) of the field strains from the ground or the clinical cases are resistant to erythromycin (Fortier et al. 1998; Fines et al., 2001)

West Nile virus

West Nile (WN) fever is a mosquito-borne flavivirus transmitted in natural cycles between birds and mosquitoes, particularly Culex species mosquitoes. In humans, West Nile infection is a non-symptomatic or a mild febrile illness; however encephalitis cases are reported with some fatalities particularly in old patients. West Nile Virus is also a cause of animal disease, especially in horses and birds (Castillo-Olivares and Wood, 2004; Dauphin et al, 2004; Murgue et al, 2001).
West Nile virus was first discovered in 1937 in the blood of a native woman of the West Nile province of Uganda who at that time was suffering from a mild febrile illness. Since then, both sporadic cases and major outbreaks of West Nile fever have been reported in Africa, Middle East, Europe and Asia and many aspects of West Nile infection have been well documented since the early 1950's in Egypt and in Israel, in the 1960's in France, and in the 1970's in South Africa. However during the last six years many reports about West Nile virus have been published, because of outbreaks occurring in Romania, Morocco, Italy, Russia, Israel, France… but more especially with the discovery of the virus in North-America in 1999. Therefore, WN fever has recently become a major public health and veterinarian concern.

The confirmation of WNV infection can be made directly by identification of the virus or indirectly by testing for antibodies in clinical specimens that include post-mortem tissues, cerebrospinal fluid, whole blood or serum. In the USA, the diagnosis of West Nile disease is clinically based on a positive IgM capture ELISA at a titer > or = 1:400. IgG titers are not very useful in the acute stages, and in addition, interpretation of IgG titers is confounded by vaccination which is widespread in the USA.

Detection of WNV in field cases in horses is hampered by the typically short duration and low level of the viraemia in horses. Negative virus detection test results should thus never be regarded as evidence of absence of WNV.

Virus isolation can be attempted from cerebrospinal fluid (CSF), blood or tissues in Vero, RK-13 cells or mosquito cell lines (Ostlund et al, 2001). However, cytopathic effect is not always evident, especially in mosquito cells, and indirect immunofluorescence using a monoclonal antibody (MAb) of high specificity is necessary to confirm the presence of and / or to identify virus isolates.

Alternatively, the presence of the virus can be confirmed by nucleic acid detection. A sensitive and WNV-specific reverse transcription and nested polymerase chain reaction method has been used successfully as a diagnostic tool in clinical samples from suspected cases of WNV encephalitis (Johnson, et al, 2001). The complete genome sequence of multiple WNV strains has been determined. This information on the molecular blueprint of the various virus strains aids the development of highly specific molecular based diagnostic tests. Both classical PCR and real-time-based tests (Taq-Man, NASBA and LightCycler) have been successfully applied to biological samples.

**Equine influenza**

Influenza A is a common respiratory infection of horses, and rapid diagnosis is important for its detection and control (Van Mannen, 2002). The in-clinic kits [Influenza A Antigen Detection Kit] are fairly sensitive and can be safely used in the field. Sensitive detection of influenza currently requires viral culture and is not always feasible. The polymerase chain reaction (PCR) was used to detect DNA produced by reverse transcription of equine influenza in stored nasal secretions, vaccines, and allantoic fluids (Donofrio et al, 1994).

Molecular diagnostic tests that allow timely and accurate detection of influenza are already implemented in many laboratories. The combination of automated purification of nucleic acids with real-time PCR should enable even more rapid identification of viral pathogens such as influenza viruses in clinical material. The recent development of DNA microarrays to identify either multiple gene targets from a single pathogen, or multiple pathogens in a single sample has the capacity to transform influenza diagnosis. While molecular methods will not replace cell culture for the provision of virus isolates for antigenic characterisation, they remain invaluable in assisting our understanding of the epidemiology of influenza viruses.

**Equine viral arteritis**

This ubiquist and sometimes serious viral disease, is checked regularly as well for natural service or semen (IA) than as at the time of evocative clinical signs (hyperthermia, cutaneous papules, limb swelling…) The epidemiologic reservoirs of the virus which are potentially the healthy shedders after a contaminating contact, are the subject of serological testing in particular using the official method which is the seroneutralisation test. Recent work (Newton et al., 2004,) showed that the repeated vaccination of the horses induced cytotoxic effects of the sera, prejudicial to seroneutralisation testing. A Workshop
took place in Kentucky in October 2004 to give a progress report on the improvements necessary for serology (ELISA methods) and the world epidemiologic situation. The genome of this small RNA virus being well-known (Balasuriya et al., 2004), the methods of genic amplification allow today the sequencing of strains regularly isolated from the field (respiratory, genital, abortive form) in order to prevent the possible appearance of wild strains which could show the same characteristics of virulence as the historical “Bucyrus” strains which caused serious epidemics of abortion in the United States. Many countries organized survey networks in partnership with specialized laboratories, to this end. In France this network (R.E.S.P.E) is located in Normandy in the middle of the major horse breeding farms area.

Parasites

After the decline, during the last 25 years, of large strongyle infections as a result of widespread use of modern anthelmintic compounds, the clinical importance of small strongyle infections has become underlined and these pathogenic nematodes have been recognised as an important cause of gastrointestinal diseases in horses including weight loss.

Cyathostome infections are ubiquitous in grazing horses and consequently, these species are now considered as the major targets of equine parasite control programs for mature, well-managed horses. So, recent researches aim at developing molecular methods of species identification from eggs or larval stages.

Intergene spacer (IGS) region DNA sequences have also been used to derive species-specific molecular probes for six common species (Hodgkinson et al., 2001). These probes have subsequently been developed for use in a high-throughput PCR-ELISA system (Hodgkinson et al., 2003). This PCR-ELISA system was recently used to determine the species present in cyathostome L4 populations from diarrheic faeces of horses suffering from larval cyathostomosis.

Currently, there is no specific laboratory method to certainty diagnose cyathostome larval infection. An immunodiagnostic assay was developed to detect the presence of small strongyle mucosal larvae. Two antigen complexes (20 and 25 kDa) were identified as specific to mucosal larvae (Dowdall et al., 2002) and appeared particularly useful as markers of pre-patent infection. The 25 kDa antigen complex has also diagnostic potential for estimating mucosal larval burdens (Dowdall et al., 2004).

Equine tapeworm infection has been recognized as a cause of spasmodic colic or ileal impaction colic and is frequently associated with lesions at the ileo-caecal junction. The coprological diagnostic methods are easy to perform but lack sensitivity: 11-61% according to validation studies (Meana et al., 1998; Proudman and Edwards, 1992).

Serological methods (ELISA) were developed using excretory/secretory antigens of 12/13 kDa to detect IgG(T) responses. Immunoblotting studies demonstrated no cross reactivity between A. perfoliata 12/13 kDa antigen and the protein antigens of other equine helminths. The test diagnostic sensitivity was 68% and the specificity 95%. The ELISA results correlated well with the infection intensity (Proudman and Trees, 1996).

This test could have important clinical application as the risk of tapeworm-associated colic is linked to the infection severity. The anti-12/13 kDa IgG(T) ELISA is currently commercialised in UK (Diagnosteq).

References


