Equine Infectious Anemia Workshop

February 14-17, 2011

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General Comments:

At the request of Dr. Gian Luca Autorino, we (Drs. CJ Issel and RF Cook) traveled from our home base at the Gluck Equine Research Center at the University of Kentucky, USA, to the Istituto Zooprofilattico Sperimentale del Lazio e Toscana, subsequently referred to as IZS. Dr. Autorino is the Head of the Reference Center for Equine Diseases at the IZS and we have collaborated with him on their work on EIA since the discovery of cases in Italy in 2006. We were invited specifically because of our experience in the virology, pathogenesis, transmission, diagnosis and control of the equine lentivirus known as equine infectious anemia (EIA) virus. EIA has been the major focus of the scientific career of Dr. Issel, who has published widely on the subject since 1974, first at Louisiana State University, and at Kentucky since 1990. Dr. Cook joined Dr. Issel in Kentucky in 1991 and has performed the majority of virus genetic studies of the research team. Recently, they have participated in the control and analysis of data from outbreaks of EIA in Ireland and Italy in 2006, and continue to share their data, experience and guidance on all aspects of EIA, with a special focus on diagnostic assay development and deployment (Issel) and in the comparative genetics of EIA virus sequences and isolates from countries in the European Union and in South America (Cook). Dr. Issel submitted his CV for review and will make PDF copies of his publications available on request.

The meetings with Dr. Autorino and his staff of the IZS working with EIA, collaborators from other groups (IZS Reference Center for Equine Infectious Anemia, Department of Pisa), and representatives from the Animal Health Unit, Ministry of Health spanned from Monday Feb 14 through Thursday Feb 17, 2011. An outline of topics and presenters is given in Appendix A and a complete list of participants in Appendix B. The meetings were held to review the current state of diagnostic findings from the National Surveillance program in Italy since 2007, the utility of the Competition ELISA test developed in situ by IZS staff, and to review plans for additional efforts of the IZS. The IZS staff has also been using an immunoblot protocol developed by our laboratory in Kentucky in the surveillance program and the results were discussed with the express purpose to standardize and correlate findings, especially with samples from our laboratory that pose greater challenges for diagnosis. Additional discussions were held on two main subjects: standardizing and validating serologic tests for wider acceptance and deployment internationally, and development of accurate and specific tests for the detection of the nucleic acids of EIA virus in widely used PCR procedures. PCR based techniques have the potential to become important adjuncts in the routine diagnosis of EIA virus infections because they offer a direct means for identifying viral structural components, if test results can be validated against known accepted techniques.

We have organized the report into an introduction with the major findings and recommendations listed first, followed by a detailed description of the meeting, where the major findings are restated and explained.

We offer the following list of highest priority items and recommendations from our perspective that, in our opinion, would further establish the IZS position as an important player in EIA internationally. The kits produced by IZS appear to be equivalent or better than those marketed in the United States and the technical competence of the staff excellent. Results of equid samples in all approved test kits were identical to ours. The meeting led to better alignment of interpretation of reactions in immunoblot tests developed by our laboratory and to excellent discussion for future work by all parties.

1. Select the most appropriate reference laboratory for EIA with recognized proficiency, expertise and experience in testing for EIA, which should be an acknowledged leader in ensuring the highest standards of quality control and with accepted international credibility among the scientific community. This is important for Italy at this time. The Istituto Zooprofilattico Sperimentale del Lazio e Toscana (IZS) in Rome is the logical choice for this role as National Reference Laboratory because of its demonstrated competence, expert leadership, and technical infrastructure. Their participation in the EU workshop on EIA in 2010 was evidence of their international recognition and reputation in EIA surveillance and control. Furthermore, this laboratory has developed and now oversees the manufacture of all diagnostic assay kits used in the EIA National Surveillance Program. Therefore the consolidation of responsibility for test kit development, for monitoring and confirming results of field application of the kits, and for directing future EIAV research projects is likely to provide significant savings of time and materials, and help standardize results.

2. Establish a three-tier EIA testing infrastructure in which all routine samples are screened in local laboratories using IZS-produced ELISA test kits with positive samples sent for confirmatory testing with the IZS ELISA and AGID test kits at a regional laboratory. The third tier of the system is the National Reference Laboratory whose role is to perform additional analyses on samples with test results in the regional laboratory that are not concordant. Their testing should include the use of additional commercial ELISA kits to minimize the bias from false-positive ELISA test results on one kit. The National Reference Laboratory should also monitor the performance of all laboratories involved in EIA screening as it is clear from both this meeting and a 2010 European Union EIAV Workshop that agreement in test results between laboratories is less than ideal.

3. Adopt strict uniform guidelines for determining the status of an equid as "POSITIVE for EIA". At the current time with the knowledge accumulated to date, our recommendation is that all equids be considered Positive if they have a positive AGID test result or if they have ELISA positive/AGID negative test response **and** positive immunoblot test result, defined as serum antibody binding to at least 2 of the 3 major proteins (gp90, gp45, and p26) at levels equal to those of a reference weak positive serum (such as the horse Flicker) when tested at the same dilution.

4. Publish findings on the utility of the Competition ELISA test developed by the IZS staff in Rome and the use of the immunoblot test employed in the National Surveillance Program. As part of this exercise, validate to the satisfaction of the appropriate sanctioning bodies the utility of the C-ELISA and immunoblot tests in combination with the AGID test to improve the diagnosis of EIA in field situations.

5. Promulgate widely your findings and assist in making the EU more effective in their diagnosis of EIA. This could be accomplished by helping sponsor a workshop on laboratory diagnosis of EIA as we discussed and in which we would gladly participate if invited. Your initial efforts to share surveillance findings on methods are a perfect entrée to this priority.

6. Continue to develop and validate PCR techniques for confirming the routine diagnosis of EIA. However this effort should not be duplicated in different government laboratories. PCR-based assays should be designed to detect EIAV RNA in plasma and proviral DNA in monocytes and results compared to determine which assay provides the best correlation with all current serologic diagnostic assays. In addition, attempts should be made to validate PCR results with virus isolation attempts in equine monocyte derived macrophage cultures and/or in horse inoculation tests.

7. Continue to accumulate data on the distribution and source of new cases of EIA. This type of data could help define where control efforts should be focused based on sound risk assessment.

8. Continue to generate data on equids naturally infected with EIAV and their responses to infection. The results from initial studies with mules and immunosuppression are most interesting and provide a useful platform for the refinement of serologic and nucleic acid diagnostic methods, especially from equids with AGID reactions that pose challenges.

9. Design and conduct studies to evaluate the risk posed by equids coming into Italy from other EU member states. It must be designed carefully to evaluate risk compared to other similar studies of intra-Italy movement.

The detailed analyses follow.

The AGID (or Coggins) test:

The AGID test reagents and test kits assembled by the IZS staff for use in Italy appear to be at least equal in sensitivity to the commercial test kits we are familiar with in the USA. The efficacy of these reagents was investigated using a panel of previously characterized serum samples from Kentucky. These included serum harvested from horses at various time-points following infection with modified laboratory strains and problematic field samples submitted for analysis by immunoblot testing to the Gluck Center because of its status as the Kentucky EIA Reference Laboratory. The results of this testing demonstrated the AGID test kits produced at IZS contained high quality reagents, capable of producing sharp single precipitin lines. In all cases there was concordance between AGID testing performed at the Gluck Center and at IZS (presented in Table 1). As expected, those samples previously determined to have very low levels of antibody to EIAV proved a challenge for accurate analysis by the IZS staff by AGID testing alone (Table 1). This demonstrates that while the AGID test has excellent specificity, the relatively large amounts of antibody required to form visible precipitin lines place severe constraints on its overall sensitivity. Furthermore, interpreting weak AGID reactions is subjective and dependent on the visual acuity of the operator. Therefore, AGID testing can lead to horses being released because of the reporting of false negative results. Consequently, we are urging wider use of ELISA tests because of their inherent higher sensitivity and objective test result reporting.

We feel that the AGID test reagents used by IZS are set at approximately the optimal concentrations to generate a sharp line of precipitation while being as sensitive as possible. Additional testing should be conducted by making additional dilutions of reagents to see if sensitivity can be increased while still retaining a sharp line for adequate interpretation. In the past, we tested new kits with recombinant p26 antigens when first released in the USA and found that about a 20% dilution of antigen was needed to retain a Positive interpretation with serum from Flicker, our reference weak positive serum. It will be of interest to see if such dilutions can be made with these kits, but based on our initial impressions further dilutions may render the reference positive line less than optimal. It is worth doing especially as the AGID test becomes utilized more as a confirmation test for ELISA Positive samples. Making the AGID test as sensitive as possible will reduce the rate of samples with discrepant results in AGID and ELISA tests, and lead to increased confidence from the international community. The increased sensitivity of the AGID test, used only in the hands of the referral and reference labs in the three-tiered system described below, will be more acceptable as interpretation by highly trained and experienced staff can be more uniform and standardized. One thing that might also help would be the use of the large well template used in the 1970's for

confirmation testing by AGID because, in our opinion, that template facilitates more uniform interpretation of samples with low level of antibody against the p26 antigen.

ELISA tests for EIA:

There is universal agreement that any equid sampled for EIA testing and found Positive in ELISA tests must be confirmed by AGID testing, because the AGID test is the only serologic test proven to correlate with horse-inoculation test results for EIA virus. Results of extensive studies in our laboratory and confirmed by numerous field samples suggest that a low percentage of equids will escape detection if only AGID testing is performed. Therefore we have advocated using the more sensitive ELISA test first. Results to date in the surveillance program in Italy appear to confirm this finding.

Surprisingly, the results presented on the surveillance testing in Italy indicated a higher than expected rate of **ELISA positive – AGID negative samples**, in the neighborhood of 10% of the AGID positive samples encountered. The following discussion is offered to put this finding into perspective.

First, the samples we provided proved to have results with the IZS ELISA kits that correlated highly with the ELISA results from ELISA test kits marketed in the USA (see Table 1). In initial pilot studies in the USA, it was estimated that up to 30% of test positive horses may be missed by AGID-only testing. In fact, when an ELISA-test-first strategy has been employed as a routine, less than 10% additional cases have been detected. Our recommended ELISA test strategy includes, at the referral laboratory, using multiple (3 or more) ELISA test kits from different manufacturers (4 are currently produced) before testing by AGID because the false positive ELISA test results generally are kit-specific, and additional testing may not be needed. In the proposed three-tier system, only samples confirmed as Positive by 2 or more ELISA test kits require testing by AGID.

The vast majority of field samples tested by ELISA and followed by AGID have results in concordance, i.e., both Negative or both Positive. A low percentage of samples have results that do not agree and different mechanisms could be responsible for these discordant serologic results. Protocols to distinguish between these mechanisms require obtaining follow-up samples and the adoption of sequential or tiered testing procedures. **Several of the most likely explanations for the discordant results are listed below.**

A. Recent Exposure to EIA virus

Seroconversion following exposure to EIA virus requires clonal expansion of specific antibody producing B-cell populations and is not an immediate process. It is likely therefore, that for at least some time during this period of expansion sufficient antibody will be produced for detection by EIA-ELISA formats but not in less sensitive AGID reactions. Eventually recently exposed equids are expected to become seropositive in all test formats. Under carefully controlled experimental conditions this generally occurs within 45 days post-infection, although in some unsubstantiated field cases incubation periods as long as 157 days have been reported.

B. Low Titer EIA virus Antibody Production

A very low percentage (estimated at less than 1% in the US) of EIA cases are characterized by persistently low antibody titers to the virus. We think this results from very low levels of antigenic stimulation possibly because these individuals are capable of exerting exceptional control over EIA virus replication or they are infected with a strain possessing poor replicative potential. Persistent low antibody titers to EIA virus have been documented in a number of cases in our laboratory, e.g., the horse Flicker that we use as a reference weak positive serum. Serum samples from this individual consistently produced equivocal results in AGID although they were clearly interpreted as positive in all EIA-ELISA formats and in the immunoblot test (see below and Appendix C). Most importantly, EIA virus infection in this horse kept in strict isolation was demonstrated by inoculation of blood into a seronegative recipient pony. However, the fact that virus was transferred in only one of six attempts graphically illustrates the very low levels of circulating infectious virus present in these rare individuals.

C. EIA Virus Core Antigen-Restricted Reactors

We have identified some equids whose serologic reactions are restricted to just the major core antigen (p26) of EIA virus. This reactivity is generally of a low titer and therefore most often detected in EIA-ELISA formats, although very rarely serum samples from some individuals will produce weak positive reactions in AGID. The most important characteristic of these equids is that serological reactivity remains restricted to p26 over time and so antibodies to the other viral structural antigens are never detected. Furthermore, the antibody titers to p26 usually diminish as time progresses. As a result of this very restricted serological reactivity and the fact that retroviral core antigens possess cross-reactive determinants, we believe such animals are not infected with EIA virus but have instead been exposed to another viral entity. Further studies are required to identify the virus or viruses responsible for restricted p26 reactions.

D. Laboratory Errors

It was reported to us that a higher than expected percentage of the field-positive ELISA tests initially identified by the satellite EIA diagnostic facilities in Italy were not confirmed following retesting at the reference laboratory. Although this seems unusually high, a similar rate is encountered in the United States, indicating that in many countries the first-level diagnostic capability on routine samples is lower than desired. Unfortunately, almost all EIA diagnostic proficiency tests used internationally are announced prior to their implementation and laboratory personnel exert extra care in conduct and interpretation of results. Of special interest is the increased sale of ELISA test kits in the USA coincident with the annual check test, suggesting the use of additional laboratory procedures to verify results prior to submitting them. Therefore, operators are acutely aware they are in a test situation and routine day to day proficiency is not adequately examined. This was recently demonstrated in the state of Oklahoma when a check of routine diagnostic test results revealed a higher EIA false negative reporting rate than found with more conventional proficiency testing methods. This finding provided an added impetus to change, and prompted the state to adopt the three-tier system described below. We argue that a review of routine EIA diagnostic procedures is more instructive than proficiency testing as currently performed and the adoption of such reviews could result in more objective initial reporting along with higher levels of concordance between satellite and reference laboratories.

Case Studies:

The samples we brought for testing by IZS techniques and personnel represent all 4 of the categories listed above. The results from IZS were comparable to ours and this exercise was successful in sharing strategies to try to explain and minimize the impact of these cases. The only category subject to control is Laboratory Error which can be reduced considerably by ELISA-first testing. The other 3 are dependent on natural phenomena and require additional study to help resolve, e.g., additional testing at 14-21 days to minimize the impact of false negative results because of recent exposure (category A), and immunoblot testing for horses in categories B and C.

During the meeting we discussed our findings on one horse "Nora" in the USA that, after extensive laboratory testing, was regarded as exposed to EIA virus but for which we stated that there was no evidence of active infection. Samples collected from this horse over 5 months each had weak reactivity to both gp90 and p26 in immunoblot tests, but below the staining intensity of Flicker tested at the same dilution. The medical history of this horse included intentional inoculation with blood from a known EIA virus carrier, and field exposure to EIA test-positive horses for multiple months. Our judgment on this case was based on negative laboratory tests by AGID and ELISA, confirmed in our laboratory, and immunoblot staining less than that of Flicker that was stable through time.

The Three-Tier Laboratory System:

In our opinion, diagnosis of EIA could be improved by using a three-tiered approach, promulgated by our laboratory and endorsed by the US Animal Health Association's Infectious Diseases of Horses Committee (Appendix E). In the United States, it is envisaged that all first tier testing will be performed in laboratories using one of the commercially available, USDA approved ELISA test formats, as these are more sensitive and less susceptible to subjective interpretation than AGID. All positive test samples are then submitted to a second tier regional laboratory for confirmation by multiple EIA-ELISA test kits (as the false-positive reactions that can occur in any one kit are generally not reproduced in all of the other ELISA formats), and by AGID. Samples deemed positive are reported by the regional laboratory. Samples whose results in ELISA and AGID tests do not agree are then submitted to the third tier reference laboratory for final confirmation and additional testing by immunoblot if needed. Please see Appendix F for a more detailed description of a preferred decision tree for use in the three-tiered system.

The Immunoblot test for EIA:

The main advantage of the immunoblot test for EIA diagnosis is that it permits serological detection of multiple viral antigens including the highly immunogenic surface unit (SU) envelope glycoprotein (gp90). In this test the overwhelming majority of EIA virus infected equids recognize at least three viral antigens including SU, the transmembrane (TM) envelope glycoprotein (gp45) and the core antigen (p26). This distinguishes itself from the AGID and ELISA tests that only detect antibody against the major core protein (p26). One format utilizes a synthetic peptide for gp45 and p26 antigen but does not distinguish between

reactions, so we regard it as an anti-p26 test, especially since the kit was modified from gp45 only because it was reported to miss a percentage of infected horses.

Therefore, cases of EIA are readily distinguishable from the few animals that possess only antibodies to the core antigen by the immunoblot procedure. Until now, the immunoblot membranes for deployment in Italy have been produced at our laboratories at the Gluck Center using a density gradient purified, fibroblast cell-adapted strain of EIA virus. It is not surprising therefore, that results in this test were identical between the IZS laboratory and the Gluck Center with the Kentucky panel of test serum samples (Table 1).

Although the immunoblot test appears to be a particularly powerful addition to established EIA serological diagnostic procedures and has been used very successfully as research tool, it has not been exhaustively tested in the field. Indeed some potential problem areas have been identified such as those animals that possess reactivity restricted to p26. These observations highlight the fact that non-specific or even cross-reactive reactions can occur and this possibility should always be taken into consideration especially when confronted with very low-levels of staining in immunoblot procedures. Rigorous selection of reference standards and uniform interpretation of field sample results is imperative. Our utilization of serum from the horse Flicker as a reference positive sample in the immunoblot procedure is based, as outlined above, on the fact that despite persistent "equivocal" AGID test reactions (see original reference, Appendix C) the horse was proven to be an EIA virus carrier in the horse inoculation test. Therefore, under ideal circumstances the EIA virus infection status of animals should be confirmed using independent diagnostic techniques before their selection as alternative reference weak positive samples for the immunoblot assay. Similarly, because of the potential for non-specific or cross-reactive binding, the EIA virus infection status should be independently confirmed in field cases producing very weak reactivity against one or even two viral antigens before a definitive diagnosis is given.

Unfortunately, additional research is required to investigate the potential of sensitive and specific alternatives to the currently available EIA serological diagnostic techniques. At present, viability of equine monocyte derived macrophage cultures is not consistent enough for reliable conventional virus isolation, current viral antigen detection methods are too insensitive and PCR-based techniques for the detection of viral nucleic acids while promising are still in their infancy (see below). Although the horse inoculation test is relatively sensitive, it may, as demonstrated in the case of Flicker, require multiple, costly blood/plasma transfers to provide an accurate diagnosis. Furthermore, ethical issues posed by this technique are likely to preclude its use in many countries.

Therefore, until effective alternative confirmatory testing procedures are developed, the immunoblot test provides an independent observation to help resolve the status of equids Negative by AGID and Positive by ELISA. From this discussion, it is evident that uniform standards should be developed for the interpretation of results. As indicated above, within the United States the immunoblot testing has been used successfully to evaluate the small percentage of samples that are seropositive in one or more EIA-ELISA formats but reported negative in AGID. In all similar serological cases where exposure to EIA virus has been confirmed, such as in the horse Flicker or equids infected experimentally with highly attenuated viral strains, there is readily discernable reactivity to at least gp90, gp45 and p26 in the immunoblot assay. As the vast majority of AGID negative-ELISA positive samples from the surveillance effort in Italy also react strongly with these antigens, it is suggested that "serum antibody binding to at least 2 of the 3 major proteins (gp90, gp45, and p26) at levels equal to those of Flicker when tested at the same dilution" be defined as the minimum

requirement for seropositivity in the immunoblot test. The currently available evidence suggests that accepting this definition as the standard for routine use will virtually eliminate the potential for misdiagnosis resulting from cross-reactive or non-specific interactions.

Two particularly problematic cases arising from the Italian National EIA survey (one of which was a horse called Rocket) were discussed at length. Serum samples from both cases were interpreted as negative by AGID, although one produced an unusual line of non-identity. Both samples showed some competitive reactivity in the IZS ELISA test. However, as a result of the unusual AGID reaction and minimal activity in the IZS ELISA tests, samples from these cases were tested by immunoblotting where they both produced weak perceptible reactions in regions of the immunoblot membrane corresponding to gp90 and p26, and the pattern of reactivity did not appear to change in sequential serum samples collected over several months. It is important to note that the reactivity noted by IZS staff was much lower than with the reference positive standard recommended by us. While antibody binding to two of the major viral antigens is consistent with exposure to EIA virus, the extremely low levels of reactivity observed in the two Italian samples discussed earlier could also be explained as an incidental exposure to EIA virus like the one that occurred in Nora's case (see Case studies, p6).

Scottish law differs from that in many countries in that in addition to “Guilty” and “Not Guilty” a verdict of “Not Proven” is often given. “Not Proven” implies the existence of some incriminating evidence but that it is insufficient for conviction of a defendant beyond a reasonable doubt. On the basis of currently available evidence, the EIAV status of the 2 horses in question is “Not Proven” in that they are negative in official tests and that their reactivity in immunoblot is not sufficient for us to report as positive by the standards employed in our laboratory today. This standard has been developed and refined over years of observations mainly from equids experimentally infected with EIAV and from a number of field samples, and will continue to be improved as data warrant.

We recommend that in such situations the horses should be submitted to further sampling to verify their status regarding infection by EIA virus. Quarantine should be applied to these horses until their status is definitively established in view of well-being of other healthy horses.

As stated earlier, the immunoblot test has been used extensively in our experimental studies for over 25 years but we have not exhaustively tested field samples from equids. For that reason, the immunoblot test for EIA is referred to as a “Research Test”. The data set from the surveillance program in Italy is an excellent testing ground to establish the utility of the immunoblot test for use in EIA virus diagnostic/surveillance programs. Therefore, we urge IZS to publish their findings and we would be pleased to collaborate to help “validate” the test for the international community. We have a vast repository of samples that could be used for this validation exercise.

Our studies with the immunoblot test on samples from horses on multiple continents suggest several things. First, the antigens of this cell-adapted strain of EIAV have proven to contain widely conserved determinants and be of wide utility in the serodiagnosis of EIA. Second, a percentage of equids have low level activity against materials in the immunoblot membrane at regions corresponding to p26 (seen with highest frequency) and gp90 (seen rarely). Although these could be specific reactions, on prospective study these do not appear to change. The rate against the p26 region is about 10% and may vary by geographic location.

Over the last 20 years, we have recognized less than 5 horses with specific reactions to the p26 antigen of EIAV in immunoblot, ELISA and AGID tests. These antibodies may reach high enough levels to be interpreted as very weak positive AGID test reactors. The few horses in this category have been studied by subsequent sampling and have all reverted to AGID negative status within 2-3 months of their discovery. To us this suggests exposure to a related lentivirus which did not replicate in the horse but which stimulated primary immune responses to the major core protein that cross-reacted with EIAV. The wider use of immunoblotting will undoubtedly discover more of these types of reactors and may help us develop a significant database and gain knowledge to explain their occurrence. In our experience and opinion, they are so rare that they warrant little further attention at this time. Because of these very rare p26-only reactors, we urge the further testing of all samples from equids with very weak positive AGID test reactions.

Genetic tests for the diagnosis of EIA:

With EIA, direct detection methods such as the PCR family of techniques to identify viral genetic material would be particularly valuable in cases of suspected recent infection especially in the days immediately preceding the production of antibodies. Unfortunately, the use of techniques to amplify the genes of EIAV from suspect materials is still in its infancy as a proven diagnostic tool. The main obstacles facing the widespread adoption of this technology are the potential for variation in target sequences and the low plasma-associated viral burdens frequently encountered in EIA virus infected inapparent carrier animals.

Technical considerations:

In general viral gene amplification techniques such as the family of PCR-based technologies are susceptible to genetic variation particularly when this occurs within the 3' terminus of primers or in fluorescent detection probes designed to anneal at temperatures of 65^{o}C or above. Therefore, the use of PCR-based techniques with EIA virus is complicated by the high mutation rate (approximately one nucleotide substitution per replication cycle) associated with lentiviral replication. These high rates are caused by frequent recombination events between the two genomic copies contained within each virion coupled with possession of a reverse transcriptase that is prone to mismatches and lacks proof-reading ability. However, mutations that compromise important structural or functional components of the virus will be selected against. Consequently, at least some regions of the EIAV genome are expected to be extensively conserved making them suitable targets for PCR-based methods of detection. Until recently with the exception of laboratory-adapted strains, there was very little genetic sequence information available for EIAV. Fortunately, this situation, particularly in the case of *gag* gene sequences, has improved with information from North American, South American, European and Asian EIAV strains now deposited in public databases such as GenBank. Furthermore, analysis of these available sequences suggests that it is possible to design broadly reactive PCR based tests for the detection of EIAV sequences in clinical specimens although this will probably require the use of degenerate oligonucleotide primers and in the case of some qPCR techniques degenerate dual labeled probes. Obviously, as only a small fraction of EIAV strains currently circulating in equid populations around the world have been characterized even to a limited extent, all PCR tests designed for detection of this virus will require extensive validation in the field. Indeed, given the propensity of this virus to undergo genetic variation it might be worthwhile designing at least two PCR-based

detection systems directed against different regions of the viral genome and conducting these reactions simultaneously on all clinical samples until sufficient epidemiological evidence has been acquired to justify discontinuing the practice and using only one.

Although the universal detection of EIAV strains by PCR-based techniques is not beyond the realms of possibility, a greater problem may be the low plasma-associated viral nucleic acid burdens found in inapparent carrier equids. As discussed above some equids such as the horse Flicker appear to maintain strict immunologic dependent control over EIAV replication and therefore possess extremely low titers of infectious virus in their blood. Therefore, even under the most optimal conditions the likelihood of amplification of viral sequences is low from many known EIAV carriers. This has been demonstrated in experimentally infected equids and in the field where viral sequences were not detectable in plasma using a sensitive nested RT-PCR system in many inapparent but AGID seropositive animals. However, excellent correlations were observed in the latter field study between AGID and nested PCR results when the target nucleic acid sequences were derived from blood cell DNA. Although EIAV infects blood monocytes, the virus undergoes reverse transcription to produce proviral DNA, remains dormant, and is not expressed until these cells differentiate to become mature tissue macrophages. Therefore, once within monocytes EIAV is not subjected to immune-surveillance and so it possible that in inapparent carrier equids where free virus and viral antigen expressing cells are rapidly eliminated there will be higher levels of inactive proviral DNA in blood monocytes than plasma-associated viral genomic RNA. While dormancy in monocytes offers a plausible explanation for the improved correlation between AGID and PCR assays based on proviral DNA rather than viral RNA, these observations will require independent confirmation. However, if successfully verified it seems reasonable to conclude that in non-clinical cases, future EIAV genetic detection systems should be based on DNA isolated from blood monocytes and not on RNA isolation attempts from plasma. However, a potential disadvantage of this approach is that not all retroviral proviral sequences are replication competent. Indeed, the mammalian genome is littered with countless defective proviral sequences. For example, a defective lentiviral proviral genome (RELIK), which incidentally is very closely related to EIAV, has recently been discovered in the germ line of the European rabbit. Therefore, before the routine adoption of blood cell derived DNA as the substrate for PCR-based assays it might be prudent to ensure that inheritable defective EIAV-like proviral sequences are not present in certain species, breeds or lines of equid. It is predicted such examples would be persistently PCR positive but negative serologically for EIAV.

In summary, it appears that PCR-based diagnostic assays capable of detecting the vast majority of currently circulating EIAV strains are feasible, although these assays will probably be restricted to targeting the viral *gag* gene sequences until much needed additional genetic characterization studies are completed. Furthermore, the problem of low plasma-associated viral RNA burdens in inapparent carrier equids may be circumvented by experimenting with techniques to extract blood monocyte cellular DNA containing EIAV proviral sequences. These new methodologies will obviously require extensive validation in the field before adoption as adjuncts to the more conventional serologic diagnostic procedures.

We are advocates of greater use of PCR techniques in viral diagnostics, but remain concerned about its overuse because of history and experiences with arboviruses and attendant laboratory contamination

problems. Our note of caution is highlighted and presented in **Appendix G and Appendix D**. As a result of this caution we recommend development of PCR-based detection methods for EIAV together with adoption of strict protocols to minimize false positive results. These might include the following good laboratory practices:

- Provision of dedicated areas for mixing PCR reagents where diagnostic samples and in particular molecularly cloned EIAV sequences are prohibited.
- Whenever possible all PCR reagents including water should be dispensed into single use aliquots.
- Employ multiple negative controls.
- Positive PCR results should be verified by re-testing using a nucleic acid template that has been re-isolated from the original clinical material or from a freshly obtained sample.
- Positive control samples (particularly if they are DNA based) while containing homologous primer/probe binding regions and encompassing a similar amplicon length should be easily distinguishable from viral sequences.
- All PCR products derived from diagnostic samples should be sequenced and compared with those EIAV strains (especially molecularly cloned materials) known to be present in the laboratory.

Molecular Epidemiology of EIAV Based on the Gag antigen p9

Nucleic acid and predicted amino acid sequences of the p9 Gag antigen vary by more than 50% between geographically distinct EIAV isolates. However, despite this significant capacity for variation, p9 sequences are remarkably conserved for periods up to 4 years in individually infected equids. As a result of these characteristics nucleotide sequence analysis of p9 is likely to prove to be an important tool for molecular epidemiological investigations of EIAV outbreaks. This is illustrated in the case of the 2006 EIA outbreaks in Ireland and Italy that are believed to be caused by administration to foals of similar batches of a contaminated equine plasma product. The fact the EIAV isolates from both countries possess identical p9 sequences demonstrates a common ancestry and provides strong support for the viewpoint that the two outbreaks are linked.

Overall comments on IZS application of techniques for the diagnosis of EIA:

The National EIA Survey in Italy is a considerable undertaking and IZS personnel are to be congratulated on their professionalism, dedication and sheer hard work. We have reviewed the procedures and competence of the IZS laboratory using a series of discussions on topics of interest to both parties and by comparison of results on a series of historically "difficult" samples from our laboratory in Kentucky. It is clear that IZS personnel possess a high level of technical competence and that the IZS-produced AGID and EIA-ELISA test kits are equivalent in sensitivity to similar assays marketed in the United States. In the majority of samples tested to date there is complete agreement in results between the two IZS test kits. However, in any large-scale survey it is to be expected that cases will be discovered that do not comply with the expected paradigm and a number of EIA-ELISA positive, AGID negative samples have been identified. In these cases, verification of EIA status has been conducted using the immunoblot test. As outlined above, equids that possess serum antibodies reactive to gp90, gp45 and p26 have almost certainly been exposed to EIAV and

should, as is current IZS practice, be reported as positive. In addition, based on available evidence it is reasonable to predict exposure to EIAV in EIA-ELISA positive cases that react with two antigens (usually gp90 and gp45, or rarely gp90 and p26) in immunoblot providing the intensity of staining is at least equivalent to a reference positive serum such as Flicker when tested at the same dilution. In case of lower reaction to that of Flicker, additional serial samples should also be tested if possible.

The immunoblot test has been used successfully in Italy to help determine the EIA status in EIA-ELISA positive, AGID negative cases. These results have confirmed our initial findings that the current immunoblot assay is reactive with EIAV strains circulating in different countries despite containing antigen derived from a cell-culture adapted North American EIAV strain.

Future plans by IZS staff on EIA

We were presented a very ambitious outline of possible research priorities by IZS with funding from within Italy. These ranged from the rather mundane routine diagnostic sampling to elegant models to better assess risk for acquiring EIA in Italy.

The National Surveillance Program for EIA is a major undertaking that has generated significant amounts of important information concerning the relative performance of different diagnostic techniques in diverse equid populations in terms of breed, species and mule hybrids. Most importantly, this program has and continues to provide a tremendous opportunity to gather novel information, enable re-evaluation of existing EIAV paradigms and define the limits of diagnostic detection methods. To capitalize on these opportunities, the Italian Ministry of Health has approved research projects comprising four major objectives. We present below our highest priority items and detailed comments on the major objectives.

Prioritized Items

We offer the following list of highest priority items and recommendations from our perspective that, in our opinion, would further establish the IZS position as an important player in EIA internationally. The kits produced by IZS appear to be equivalent or better than those marketed in the United States and the technical competence of the staff excellent. Results of equid samples in all approved test kits were identical to ours. The meeting led to better alignment of interpretation of reactions in immunoblot tests developed by our laboratory and to excellent discussion for future work by all parties.

1. Select the most appropriate reference laboratory for EIA with recognized proficiency, expertise and experience in testing for EIA, which should be an acknowledged leader in ensuring the highest standards of quality control and with accepted international credibility among the scientific community. This is important for Italy at this time. The Istituto Zooprofilattico Sperimentale del Lazio e Toscana (IZS) in Rome is the logical choice for this role as National Reference Laboratory because of its demonstrated competence, expert leadership, and technical infrastructure. Their participation in the EU workshop on EIA in 2010 was evidence of their international recognition and reputation in EIA surveillance and control. Furthermore, this laboratory has developed and now oversees the manufacture of all diagnostic assay kits used in the EIA National Surveillance Program. Therefore the consolidation of responsibility for test kit development, for monitoring and confirming results of field application of the kits, and for directing future EIAV research projects is likely to provide significant savings of time and materials, and help standardize results.

2. Establish a three-tier EIA testing infrastructure in which all routine samples are screened in local laboratories using IZS-produced ELISA test kits with positive samples sent for confirmatory testing with the IZS ELISA and AGID test kits at a regional laboratory. The third tier of the system is the National Reference Laboratory whose role is to perform additional analyses on samples with test results in the regional laboratory that are not concordant. Their testing should include the use of additional commercial ELISA kits to minimize the bias from false-positive ELISA test results on one kit. The National Reference Laboratory should also monitor the performance of all laboratories involved in EIA screening as it is clear from both this meeting and a 2010 European Union EIAV Workshop that agreement in test results between laboratories is less than ideal.

3. Adopt strict uniform guidelines for determining the status of an equid as "POSITIVE for EIA". At the current time with the knowledge accumulated to date, our recommendation is that all equids be considered Positive if they have a positive AGID test result or if they have ELISA positive/AGID negative test response **and** positive immunoblot test result, defined as serum antibody binding to at least 2 of the 3 major proteins (gp90, gp45, and p26) at levels equal to those of a reference weak positive serum (such as the horse Flicker) when tested at the same dilution.

4. Publish findings on the utility of the Competition ELISA test developed by the IZS staff in Rome and the use of the immunoblot test employed in the National Surveillance Program. As part of this exercise, validate to the satisfaction of the appropriate sanctioning bodies the utility of the C-ELISA and immunoblot tests in combination with the AGID test to improve the diagnosis of EIA in field situations.

5. Promulgate widely your findings and assist in making the EU more effective in their diagnosis of EIA. This could be accomplished by helping sponsor a workshop on laboratory diagnosis of EIA as we discussed and in which we would gladly participate if invited. Your initial efforts to share surveillance findings on methods are a perfect entrée to this priority.

6. Continue to develop and validate PCR techniques for confirming the routine diagnosis of EIA. However this effort should not be duplicated in different government laboratories. PCR-based assays should be designed to detect EIAV RNA in plasma and proviral DNA in monocytes and results compared to determine which assay provides the best correlation with all current serologic diagnostic assays. In addition, attempts should be made to validate PCR results with virus isolation attempts in equine monocyte derived macrophage cultures and/or in horse inoculation tests.

7. Continue to accumulate data on the distribution and source of new cases of EIA. This type of data could help define where control efforts should be focused based on sound risk assessment.

8. Continue to generate data on equids naturally infected with EIAV and their responses to infection. The results from initial studies with mules and immunosuppression are most interesting and provide a useful platform for the refinement of serologic and nucleic acid diagnostic methods, especially from equids with AGID reactions that pose challenges.

9. Design and conduct studies to evaluate the risk posed by equids coming into Italy from other EU member states. It must be designed carefully to evaluate risk compared to other similar studies of intra-Italy movement.

Detailed Comments on the 4 Objectives follow:

Objective 1. Evaluate the Screening Protocols Employed in the National Surveillance Program

A major component of this objective is to investigate efficacy of the IZS EIA-ELISA kit for initial routine screening of serum samples for the presence of EIAV antibodies. It is clear from the discussions that a considerable amount of work has been conducted in this area, demonstrating the sensitivity of the assay and comparing it to the internationally approved AGID test. As proposed by IZS personnel the "in-house" EIA-ELISA should be compared with EIA-ELISA tests currently approved for use in other countries. Completion of these studies will yield very important information that should be submitted for publication in internationally recognized peer-reviewed journals. Furthermore, on the basis of available data, the IZS EIA-ELISA is clearly suitable for use in other countries and steps should be taken to receive official European Union validation for this wider application.

A second component of this objective appears to be the adoption or development of PCR-based tests for EIA. Direct virus nucleic acid detection methods such as PCR would be extremely valuable in confirming serological diagnosis and in cases where recent exposure is suspected. Quantitative (q) or real time PCR methods would also be a useful research tool for example, enabling determination of the viral burden range in different breeds or species of inapparent carrier.

As discussed above the two major problems associated with development of PCR-based techniques for the routine diagnosis if EIA are the potential for variation in viral target sequences and low viral nucleic acid levels that may occur especially during the inapparent carrier phase. However, the current situation in Italy affords an excellent opportunity to test the extent of these problems in an actual field situation and to establish if PCR techniques can play a useful role in EIAV diagnostics. If resources permit, it is certainly worthwhile investigating these techniques although the following guidelines are suggested:

- Develop or apply of at least two distinct PCR-based assays directed against different target sequences within the EIAV genome. These assays should be tested in parallel and results compared with serological testing in AGID, EIA-ELISA and immunoblot.
- Compare RT-PCR on RNA isolated from plasma against PCR using whole blood cell derived DNA as the substrate to determine the relative abundance of viral RNA compared with proviral DNA in EIAV infected equids. The results from A and B will determine the value of PCR in EIA diagnostics and should be published in international peer-reviewed journals.
- Design synthetic or recombinant PCR positive control molecules that are readily distinguishable from all known EIAV genomic sequences.
- Instigate automatic re-isolation of nucleic acid from the original and/or fresh sample material followed by re-testing on all PCR positive samples.
- Perform routine nucleotide sequence analysis on all PCR products.
- Archive PCR positive samples for subsequent molecular epidemiological investigations.

Objective 2 To Study Risk Factors in EIAV Transmission

Methodology pertaining to Objective 2 comprises a detailed questionnaire provided to farm owners/veterinarians, testing horses imported into Italy, insect traps to monitor vector populations and experiments "to study the dynamics of viremia relative to the time of infection." Although the questionnaire will provide a wealth of useful information about the farm where cases of EIA are discovered such as number of equids, species, breed, use, pasture, housing facilities and basic management protocols it is unlikely that anyone will knowingly admit to practices contributing to the iatrogenic transmission of EIAV. Careless or reckless behavior by man such as use of extension sets or syringe needles in multiple animals provides an extremely efficient mode of transmission for this virus. Unfortunately, even in this era of sterile, disposable veterinary equipment, iatrogenic transmission was thought to be the major means of spread of EIAV during the 2006 outbreak in Ireland.

Obviously, one of the most important risk factors for the transmission of EIAV is the blood associated viral burden in carrier animals. Equids with EIA disease signs frequently have viremia titers greater than 10^6 horse infective doses $_{50}$ (ID_{50}) per ml and it has been demonstrated that a single insect can transmit EIAV from these clinical cases. However, viremia titers decline rapidly with the resolution of clinical signs thereby decreasing the risk of transmission by insects. For example, in the bovine leukemia system, between 50 and 100 fly bites are required for transmission at a viremia titer of $10^{3.5}$ per ml. As many EIAV infected equids have viremia titers well below $10^{3.5}$ ID_{50} successful insect mediated transmission from these animals could in theory, require several thousand attempts. Although there is recognition of these facts in the proposed projects, it is not clear how the "dynamics of viremia relative to the time of infection" will be investigated. As outlined above, the greatest EIAV transmission risk is posed by animals with clinical disease. However, this is usually a transient state and plasma associated viral burdens can vary by several orders of magnitude within 24 hours. Therefore, if cases of recent infection are suspected, retrospective determination of EIAV

RNA burdens in potential in contact carrier animals is unlikely to be informative about the levels of virus present when transmission occurred, even assuming this time point is identified. Instead, the principal value of such investigations might be to determine if low viremia levels always correlate with low antibody titers resulting in AGID negative/ELISA positive type reactivity.

On the other hand, there is much to learn about the "dynamics of viremia" in EIAV infected equids, particularly once they have entered the inapparent carrier stage. It is assumed, these animals exercise long-term control over viral replication such that viremia levels are maintained at a constant "set-point" level. In some cases, this set-point may be extremely low with a corresponding low risk for transmission from these animals. For example, if the viremia titer of Flicker was 1 ID_{50} per 1000ml of blood then assuming the blood volume retained on the mouthparts of a horsefly is $1x10^{-5}$ ml it can be estimated that insect mediated transmission from this horse should occur only once in 100,000,000 attempts. However, in inapparent carriers it is not known if the viremia set-point is really constant or how it varies in response to environmental stress, infection with other microbial agents that possess immunomodulatory activity, age, etc.

The use of insect traps will provide information only about the current species and density of potential EIAV insect vector populations. However, these populations will almost certainly show considerable variation with time making it difficult to predict the situation that existed at the time of transmission, assuming this time point is identified. In fact, to make such predictions would probably entail very long-term studies to correlate insect populations over time with ever changing environmental factors such as climate.

Testing equids imported into Italy will yield essential information and contribute to the validation of the serological test kits developed by IZS. Furthermore, if plasma and blood cell DNA samples from seropositive animals are archived it will provide suitable material for subsequent molecular characterization and phylogenetic studies.

Objective 3. Clinical, Immunological and Virological Studies in Naturally Infected Equids.

Descriptions of the methodologies applied to these studies were not provided. Although most EIA cases are expected to be asymptomatic at the time they are discovered it is important to document all clinical cases at least in terms of signs commonly associated with the disease such as edema, cachexia, petechial hemorrhages on mucous membranes, anemia, thrombocytopenia and febrile responses. In addition, if autopsies are performed evidence of lymphadenopathy, hepatomegaly or splenomegaly should be recorded along with the presence of stainable iron in Kupffer cells or glomerular thickening associated with immunoglobulin and complement deposition. Viral isolates from these cases will by definition have a clearly defined pathogenic phenotype that may be important in future virological investigations. In addition, these viruses will be the easiest of all isolates to characterize at the molecular level (even to the extent of obtaining complete genomic sequences) because the high viral burdens present during febrile episodes maximize the potential for subsequent amplification by RT-PCR. Therefore, steps should be taken to archive as much clinical material (plasma and/or tissues such as liver and spleen) as possible from all cases of EIA where disease is present.

Without further information it is difficult to envisage the rationale, in the context of the current IZS mission, for conducting immunological studies in EIAV infected animals. A possible exception would be to investigate how antibody responses in relatively large equid populations relate to different serological

detection methods. An example, would be to test the hypothesis that unusually low viral burdens produce low antibody levels that in turn lead to AGID negative/EIA-ELISA positive results. Depending on the future political climate regarding the control of EIA, especially in countries where equids provide invaluable motive power, verification of this hypothesis may provide a mechanism for identifying animals that represent a low risk for transmission.

At present the study of cell mediated immune responses in EIA field cases is unlikely to provide much useful information because other than a requirement for functional T and B lymphocytes the actual mechanisms that control viral replication have not been identified. In fact the possibility exists that conventional adaptive immune responses may play only a partial role in limiting EIAV replication. A number of molecules have been identified such as members of the cytidine deaminase family (APOBEC3) and the tripartite motif protein 5 α (TRIM5α) that are potent inhibitors of retroviruses and retrotransposons. In future, perhaps when more information becomes available, some thought should be given to examining expression of these molecules in individuals with significantly different viral burden set points or comparisons between horses and donkeys especially as in the latter species, EIAV replication appears to be more effectively controlled.

The proposed virological experiments are not described in detail other than the characterization of nucleotide sequences. However, information generated as a result of these collective investigations will yield a wealth of opportunities for additional phenotypic and genotypic studies on EIAV especially in the context of performing genetic substitution in infectious molecular clones. The only limitations to these will be funding and their perceived significance to the IZS mission.

Objective 4 Phylogenetic Studies and Molecular Epidemiology of EIAV

This is a very straightforward objective that will yield significant amounts of information, essential to the design of new, potentially "universal" molecular detection methods and novel vaccine constructs. Studies for this objective should probably commence with an analysis of EIAV *gag* because this is the gene for which the most published sequence information is available to assist in the design of PCR amplification or sequencing primers. Nucleotide sequence determination of Gag p9 will test the hypothesis these sequences can be used as a molecular signature to trace the progression of EIA outbreaks. Once *gag* sequences have been characterized other regions of the EIAV genome might be characterized on a progressive basis until at least in a few selected examples, complete genomic sequences have been obtained. As outlined above, molecular characterization and subsequent phylogenetic analysis will be easiest in clinical cases with high viral burdens. However, one of the most important factors involved in the success of this objective will be the ability to archive sufficient sample material.

Appendix A

Draft agenda of the EIA Meeting/workshop

TIMETABLE	2/14/2011		2/15/2011		2/16/2011	
9:30	Participants presentation (Autorino)	9:30	AGID STANDARDISATION REAGENTS METHODS (RADIAL ID) AND USE OF DIFFERENT ANTIGENS (Issel)	9:30	AIE CURRENT RESEARCH PROJECT (OBJECTIVES - Autorino)	
10:00	ITALIAN EPIDEMIOLOGY (IZSLT)	10:00	THE THREE TIER SYSTEM AND THE IMPROOVEMENT OF ELISA SPECIFICITY (Issel)	10:00	AIE CURRENT RESEARCH PROJECT (PRELIMINARY RESULTS - Autorino)	10:00.12:0
10:30	LABORATORY DIAGNOSIS AND RELATED PROBLEMS (Gasparetti/Ricci)	10:30	IB PRODUCTION PROCEDURE - STANDARDISATION AND VALIDATION ? (Issel and Cook)	10:30	CHOISE AND USE OF RECOMBINANT AND SYNTHETIC PROTEINS FOR THE DEVELOPMENT OF DIAGNOSTIC TOOLS (Issel and Cook)	
11:30	break	11:30	break	11:00	break	
12:00	CBT ELISA VALIDATION (Nardini)	12:00	CRAIE PCR DIAGNOSTC AND RESEARCH PROTOCOLS (Enrica Ricci)	11:30	TIME FOR REFLECTION OF EXPERTS FOR THE REVISION OUR RESEARCH PROGRAMMES (Issel and Cook)	
12:20	COMPARISON OF ELISA KITS (Ricci/Nardini)					
12:40	lunch	12:40	lunch	12:40	lunch	
13:30	Time for the preparation of CONSIDERATIONS FROM EXPERTS (Issel and Cook)	13:30	OVERVIEW OF AIE MOLECOLAR DIAGNOSIS AND RESEARCH (Issel and Cook)	13:30	CONSIDERATIONS AND PROPOSALS FROM EXPERTS (Issel and Cook)	
14:30	DISCUSSION (Issel and Cook)	14:00	VIRUS ISOLATION AND CULTURING FROM MONOCYTES (Issel and Cook)	14:30	PROGRAMMING OF JOINT ACTIVITIES	
		14:30	TROUBLE SHOOTING WITH OUR PCRs AND SEQUENCING (Canelli e Lorenzetti)			

2/17/2011

Entire day: Review of overall discussions with focus on initial recommendations and perspective

Appendix B List of Participants from Italy

Name	Affiliation	
Dr. Luigi Ruocco	Head	Animal Health Unit - Italian Ministry of Health
Dr. Mattia Paglialunga	Staff	Animal Health Unit - Italian Ministry of Health
Dr. Laura Gasperetti	Staff	Reference Centre for Equine Infectious Anemia, Department of Pisa - Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Erica Ricci	Staff	Reference Centre for Equine Infectious Anemia, Department of Pisa - Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Ilaria Ciabatti	Staff	Department of Biotecnology - Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Raniero Lorenzetti	Staff	Department of Biotecnology - Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Ugo Marchesi	Staff	Department of Biotecnology - Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Giorgio Saralli	Staff	Department of Latina - Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Gian Luca Autorinc	Head	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Elena Canelli	Staff	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Andrea Caprioli	Staff	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Raffaele Frontoso	Staff	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Giuseppe Manna	Staff	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Roberto Nardini	Staff	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Dr. Francesca Rosone	Staff	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana
Sr. M. Teresa Scicluna	Staff	Reference Centre for Equine Diseases, Istituto Zooprofillatico Sperimentale del Lazio e Toscana

Appendix C Publication on Flicker

31.pdf

Appendix D Publication on Outbreak in Ireland, see bottom of page 5 and page 6

179.pdf

Appendix E. USAHA IDoHC Resolution on EIA with Three-Tiered Laboratory system

UNITED STATES ANIMAL HEALTH ASSOCIATION - 2008

RESOLUTION

RESOLUTION NUMBER: 26 APPROVED AS AMENDED

SOURCE: COMMITTEE ON INFECTIOUS DISEASES OF HORSES

SUBJECT MATTER: ENHANCED EQUINE INFECTIOUS ANEMIA PROGRAM FUNDING

BACKGROUND INFORMATION:

Equine infectious anemia (EIA) has been controlled in the United States because individual states with support of their equine industries have instituted regulations which require testing for entry, movement and/or congregation, as well as quarantine of test-positive equids. Testing for EIA has been widely accepted, and today includes both the agar gel immunodiffusion (AGID or Coggins) and enzyme linked immunosorbent assay (ELISA) test formats. Each year, approximately 2 million equid samples are tested for EIA, and over the last three years, 0.01 percent of the samples were reported as positive. The true prevalence of the infection is not known. In recent years, many of the reported cases have been from states with historically low numbers of cases, and a substantial proportion of those positives were in equids not previously tested for EIA. It is assumed that a population of untested equids exists in the United States. The rate of EIA infection is expected to be higher for that population in those states with historically higher reported numbers of positive tests, such as Arkansas, Louisiana, Oklahoma, Texas and Mississippi.

In the considered opinion of experts and regulators, active surveillance should not be reduced but should be improved. Changes are needed because the traditional methods have reached their plateau, and testing in the mobile tested population greatly exceeds the actual risk. The changes deemed most appropriate are those directed toward: 1) identifying the true prevalence of the infection, 2) reducing the interval of testing where appropriate, 3) devising methods to address the untested population, with a focus on states with historically higher rates of test-positive equids, and 4) implementing a three tiered testing system utilizing sensitivity and specificity of tests in appropriate sequence for maximum efficiency.

RESOLUTION:

The United States Animal Health Association (USAHA) requests that the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), in cooperation with states and the equine industry, such as the American Horse Council, state horse councils, American Association of Equine Practitioners and breed registries, request funding to support an enhanced Equine infectious anemia (EIA)

control/eradication program. Three (3) basic components encompass:

Section A: Fund Program

1. USDA-APHIS-VS to incorporate specific elements of the Equine infectious anemia (EIA) Uniform Methods and Rules (UMR) into the Code of Federal Regulations (CFR), Title 9, part 75, Communicable diseases in horses, asses, ponies, mules, and zebras, in order to assure that only equines having negative EIA testing status are moved interstate except as described under section 6;

2. Requests funding for an enhanced EIA control program leading to eradication with new money: At -risk states are to receive focused federal funds in an eradication program; the initial funding emphasis should be in the states with historically higher rates of infection (Louisiana, Arkansas, Oklahoma, Texas, Mississippi); and At-risk states must meet certain minimum standards including: change of ownership testing, minimum 12 month negative test for interstate movement, required euthanasia of reactors (grandfather existing reactors that are isolated), individual permanent identification of tested horses, utilization of a 3-tiered testing system.

Section B: Prevalence Working Group

1. USDA-APHIS-VS should create a national EIA prevalence working group that includes representatives from all "At Risk" states.

2. The EIA prevalence working group would continue collaboration with the National Surveillance Unit (NSU), Centers for Epidemiology and Animal Health (CEAH) existing equine prevalence model for:

1 Identification of industry stakeholders;

2 Accurate equine census;

3 Accurate prevalence data;

4 Consistent case definition – herd vs. head; and

5 Address other issues as appropriate.

Section C: Diagnostic Laboratory Component

1. USDA-APHIS-VS should adopt national laboratory reporting system for accurate electronic test data.

2. Re-evaluate laboratory certification (moratorium) policy with input from state/federal regulatory authorities and National Veterinary Services Laboratory (NVSL).

3. Utilize and request funding for a 3-tiered laboratory testing system (enzyme linked immunosorbent assay (ELISA), agar gel immunodiffusion (AGID), immunoblot).

4. USDA-APHIS-VS should request funding for the NVSL laboratory system to fully support an expanded program.

Appendix F. State of the Art in Serodiagnosis of EIA January 2011

Concordance of serologic results is high

There is general consensus internationally that the agreement of results between serologic results using different approved test formats (AGID, ELISA) is very high. For that reason, both formats are approved for international use and kits are marketed by a number of manufacturers around the world. As the AGID test is the only serologic test for EIA that has been shown to be positively correlated with virus presence in horse inoculation tests, samples positive on ELISA tests must be confirmed by AGID before regulatory decisions are made. There is a growing body of evidence that indicates a minority of equids infected with EIA virus (EIAV) mounts low levels of antibodies against the virus and may be falsely interpreted as Negative in AGID tests. This could occur through human error in the subjective interpretation of the result, because the antigen content in the test kit is too high, because the sample antibody level is below that required to give a positive AGID result, or a combination of these factors. Because of this, several jurisdictions have adopted or urged the adoption of a three-tier system of diagnosis with the more sensitive ELISA test used first. The three tiers of testing include (1) ELISA tests in most labs, (2) positive samples forwarded to a referral lab where additional ELISA and AGID tests are performed, and (3) reference laboratories where additional immunoblot testing can be performed if discordant results are found at the referral lab. Please see the proposed decision tree below.

When this strategy is employed, one must be prepared to resolve the confusion introduced when samples with positive ELISA and negative AGID tests are presented. The true rate of such samples, not falsely positive by one ELISA test kit but truly from an EIAV-infected equid, is not understood well but early indications are that it may approach 10% of the AGID test positive samples encountered in some regions, especially after decades of only using the AGID test to identify carriers. With the advent of additional tests, our power in accurate diagnosis of EIA has been increased. To apply this power accurately remains a challenge, in part because of a reluctance to adopt any change in a control system that has worked well for more than 35 years. In my opinion, it is no longer acceptable to find the majority of EIAV-infected horses with our surveillance. The industry has invested billions of dollars in the control program and we have the ability to identify nearly all of the EIAV-infected horses with the technology available today.

The information presented below summarizes our state of knowledge today on resolution of samples with results in ELISA and AGID tests that are not in agreement. To our knowledge, there are no samples documented to have consistent results that are positive in AGID and negative in ELISA tests. Therefore, these are not discussed.

Proposed method for resolving the status of AGID negative – ELISA positive samples

Perform immunoblot tests after the sample shows positive reactions in more than one ELISA test kit. To date, we have not seen samples with false-positive ELISA results in multiple kits. Samples with reactions interpreted as negative but with color close to the positive cutoff point in multiple ELISA kits, however, should be investigated further (see below).

When the sample is tested by immunoblot and recognizes at least 2 of the 3 major proteins of EIAV (envelope proteins gp90, gp45 and the major core protein p26), it should be considered positive. For comparison, all the approved test kits for EIA serodiagnosis detect antibody against the p26 antigen. As samples with AGID negative - immunoblot positive reactions could be coming from animals that have received passive antibodies (colostrum, blood transfusion, or administration of plasma from positive horses), verify result with second sample at 14-28 day interval and verify through amnesis that the subject has not received medical treatment that could have involved the materials listed above. (As these materials must have originated from an infected horse, the probability that virus was also introduced must be considered.)

In the case of decay of passive antibodies obtained via colostrum from the positive mare, we would expect the reactions to first become interpreted as negative by AGID, then by ELISA, then by immunoblot. This difference can be explained by the relative sensitivity of the tests for detecting antibodies. By immunoblot, the time of persistence of antibody depends on the level in the mare and may persist beyond 12 months. Repeated sampling showing a gradual decline in reactivity with time is a good prognostic sign.

What about those with negative AGID and ? ELISA results

Over our 36 years of studying EIA, we have been privileged to observe a number of inapparent carriers of EIAV over extended time periods (several decades). In field studies at LSU and in quarantine facilities in Florida we have observed several adult horses that have gone from AGID positive to negative, and also from ELISA test positive to suspect. Samples from these horses have consistently given positive immunoblot tests. The samples, though, have always been reactive by ELISA but below the cutoff line for interpretation as positive. In these cases, the reactions in all the ELISA test formats showed comparable decreases in reactivity over time. Samples from some of the horses collected later became stronger reactions in ELISA and/or AGID test formats and could be interpreted as positive. This could be caused by reactivation of latent virus cell reservoirs in the carrier horse. In all of these cases, however, infection from other sources could not be ruled out (these horses had pasture mates also infected with EIAV).

These types of reactions have been seen with higher frequency in horses that have been administered our live virus vaccine for EIA; the virus has a deletion in its S2 gene that reduces the ability of the virus to replicate in horses. These cases were as described above: negative by AGID and some were reactive on the ELISA test kits used but interpreted as negative per spectrophotometric/visual comparison with the appropriate controls. These samples were also positive by immunoblot as described above.

Overall assessment – Please see flowchart on next page

It appears that a minority of horses and other equids infected with EIAV in the field have low levels of antibodies against the virus through time. Our hypothesis in these cases is that they control virus replication very effectively, suggesting that continued antigenic stimulation may be necessary to maintain high antibody levels. In earlier studies we have shown that not all inapparent carriers of EIAV have 1 infective dose of the virus in 1ml of whole blood and we cannot rule out the possibility that some horses may clear EIAV from their bodies. That, however, would be impossible to prove. Additional testing with horse inoculation tests for infectious virus or PCR tests for virus genetic material may help prove the continued presence of EIAV in the horse. There is unanimity of opinion among animal disease regulators that once a horse is infected, it remains infected for life. Although the risk posed by individual infected equids cannot be accurately documented and is thought to be low in most inapparent carriers, it cannot be predicted and is known to change through time. **Therefore, in my opinion those horses with the negative AGID, ? ELISA (in more than one format) and positive immunoblot results should be isolated and treated as carriers of EIAV.**

Proposed Decision Tree for Serodiagnosis of EIA using the Three-Tiered System January 2011

1st Tier Field Lab

Test sample in approved ELISA test format

Negative		**Report as Negative**
Positive	**Retest to verify; if Negative**	**Report as Negative**
	If Positive, forward to 2nd tier	

2nd Tier Referral Lab

Test sample in same and additional ELISA test formats

Negative		**Report as Negative**
Positive in only 1		**Report as Negative**
Positive in ≥2 ELISA kits from different manufacturers		
	Perform AGID test	
	If Positive	**Report as Positive**
	If Negative, forward to 3rd tier	
Suspect in all	**Perform AGID test**	
	Note result and forward to 3rd tier	

3rd Tier Reference Lab

Test sample in all formats to confirm

If confirmation, test by immunoblot

If gp90, gp45 and p26 are recognized	**Report as Positive**
If 2 major proteins are recognized (more common outside US)	**Report as Positive**
If only p26 is recognized (exposure to related lentivirus?)	**Report as Negative**

Notes: If this algorithm is followed, we would expect >99.99% of field samples to be resolved without involving the 3rd Tier laboratory.

Respectfully submitted,
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Appendix G. Reprinted from the Equine Disease Quarterly, 12(1), January 2003

The technology for harnessing the innate specificity of nucleic acids for disease diagnosis has advanced considerably in recent years, as discussed in the article entitled Nucleic Acid Based Tests in Disease Diagnosis in this issue. The tedious purification techniques of the past have been replaced by automated micro (even single cell) extraction procedures and visualization with sophisticated fluorescent dyes. Nucleic acid sequencing and polymerase chain reaction (PCR) techniques that detect as few as five molecules have largely supplanted relatively crude methods such as hybridization and restriction endonuclease fragment analysis. The hybridization protocols of today are frequently in association with microarrays that permit the expression of thousands of genes that can be analyzed simultaneously with robotic operation.

The technical ability to amplify bits of unique genome material of pathogens has broadened our scope in diagnostics. What are the strengths and weaknesses of such sensitive procedures? When are they most appropriate, and when are they misleading? A few examples follow.

Two of the most powerful applications of PCR have been with pathogens that pose a risk to humans, namely HIV and West Nile encephalitis. By amplifying subgenomic stretches of the pathogen, the work can proceed under lower levels of biosecurity once the nucleic acid is extracted. In HIV, PCR is widely used to monitor viral burden (number of viral RNA copies) in blood of HIV-infected patients through time and to monitor the effectiveness of antiviral drug therapy. In the former, the assays are directed at highly conserved regions of the most conserved gene. In the latter, application of the PCR reaction is followed by sequencing to monitor for drug-resistant mutants. For West Nile virus (WNV), the PCR amplification of bits of the viral genome has permitted human and veterinary diagnostic laboratories to isolate WNV and participate in the surveillance of West Nile virus in North America.

A weakness of the PCR approach to diagnostics is also its strength: it is sensitive enough to theoretically amplify one copy of the pathogen genome. The likelihood of amplifying the genome of inactivated or defective pathogens under those circumstances is high. The incredible sensitivity of PCR-based techniques means there is also a constant threat of cross-contamination with the possibility of generating false-positive results. In any case, the presence of a positive PCR signal must be carefully assessed by "real-time PCR" where a copy number of the pathogen genome can also be addressed. The powerful nucleic acid techniques require expensive equipment, often beyond the reach of small laboratories. Highly trained personnel must be employed to ensure the optimal handling of sample materials to prevent degradation and contamination. Despite these constraints, the potential for such techniques in medical/veterinary diagnostics is exceptionally high.

One of our major fears about the widespread adoption of nucleic acid techniques is that they will lead to a mindset or culture in which isolation, identification, and basic research on pathogens is considered too mundane for funding. A healthy balance must be struck between the contemporary and traditional camps to continue to make progress in the identification of known, emerging, and novel pathogens that leads to their effective control.

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