Performance and trends of the results of the Interlaboratory Trials held between 2002-2017 for the serological tests employed for the diagnosis of Equine Infectious Anemia

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SUMMARY

The aim of this paper is to analyse the trend of the Interlaboratory Trails (IT) for serological techniques used in the diagnosis of Equine Infectious Anaemia (EIA), organized by the National Reference Centre for EIA between 2002-2017. The participation of numerous national laboratories and the long time interval considered gives greater value to the data obtained. The increase in the samples to be tested as prescribed by the legislative provisions, and the requirement to participate with a satisfactory outcome for accreditation purposes has made this tool important for verifying the technical and diagnostic competence of the laboratories. The performances obtained by the laboratories demonstrate the efficiency and the reliability over time of the diagnostic system, which plays a central role in the surveillance activities.

Overall, although the whole diagnostic system has an acceptable sensitivity, the ELISA presented higher concordance levels and better reproducibility than the agar gel immunodiffusion (AGID). The implementation, since 2007, of the regular serological testing for EIA requested the use of highly sensitive techniques, such as the ELISA; this assay indeed is capable of detecting about 20% more positives samples than AGID and can more easily detect weak positive sera that, in an advanced stage of eradication, constitutes the majority of the positive samples and are difficult to diagnose with the previous test, since those with a strongly positive reaction have been already recruited and removed through previous surveillance activities. The high number of laboratories that immediately participated in the IT for ELISA, on its introduction, reflects its diagnostic value owing to its high sensitivity, underlined by the reduced percentage of errors obtained for the positive sera. This makes it an effective method, in support of the eradication of infection to be applied during the screening phase of the national surveillance program, to recruit sera not detected in AGID.

KEY WORDS

Equine Infectious Anemia; Interlaboratory Trials; ELISA; AGID; performance.

INTRODUCTION

The inter-laboratory trials (IT) includes the organization, execution and evaluation of measurements or tests conducted under similar conditions on the same samples from two or more laboratories.

Participation in the IT is fundamental for laboratories that use tests accredited by official bodies (ACCREDIA for Italy)[1,2,3], since accreditation and its maintenance are subject to participation in comparison circuits with satisfactory results[4,5].

The IT allows to continuously evaluate and monitor the technical competence and the performances of the laboratories for specific tests or measurements; through the circuit it is also possible to compare the different methods used and define their effectiveness.

The UNI CEI EN ISO/IEC 17043[6] specifies the requirements of the organizing bodies[1] that generally propose criteria of acceptability of the results and, at the end of the trial, return to the participants a statistical elaboration of the results obtained.

Although IT offers a snapshot of the activity of the laboratory and does not allow a complete evaluation with respect to the maintenance of the requirements of the accreditation program, it is a very valid tool in aiding to identify problems that can compromise the quality of the test results[1] and subsequently implement improvement actions where necessary.

According to the activities foreseen by the Ministerial Decree October 4, 1999[7], since 2002 the National Reference Centre for Equine Infectious Anaemia (CRAIE) has organized, with regular frequency, the IT for the different techniques used for the serological diagnosis of Equine Infectious Anaemia (EIA), to guarantee that the national laboratories authorized to carry out these tests, the possibility of obtaining and maintaining the requirements for accreditation. From the beginning, numerous laboratories of the national network of Istituti Zooprofilattici Sperimentali (II.ZZ.SS.) and of other national and foreign laboratories that perform the serological diagnosis of EIA have participated in the IT. Initially, the circuit was intended for the Agar Gel Immunodiffusion test

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(AGID) only, performed according to the Ministerial Decree (M.D.) 12/04/1976 (Coggins test)[8] employing double layer agar. Since 2006, also the AGID technique described in the OIE Manual (AGID OIE)[9], using a single layer agar plate, was evaluated in the IT. From 2012, the frequency of the circuit, initially annual, was then run biannually and at the same time the evaluation of the ELISA technique was introduced, which gradually replaced in many laboratories the AGID for the diagnosis of EIA in the screening phase.

The purpose of this work is to analyse the performance of the IT organized by the CRAIE between 2002-2017 with respect to the participation of the laboratories, the techniques used in the serological diagnosis of EIA and the performances obtained for each method. The value of the data obtained from the comparison is significant in view of the involvement of numerous laboratories distributed throughout the national territory and the wide time interval analysed.

MATERIALS AND METHODS

Organization of IT

Within each IT the participating laboratory received the same panel of sera but with a different identification of the samples[3] together with a descriptive operational protocol of all the phases, including detailed indications on: serum management, execution of the test, timing and method of sending the results. The elaboration of the results received from each participant was carried out by CRAIE and relative to this the laboratories received a detailed report on their performance with the data presented anonymously[3]. The IT panel was prepared starting from positive and negative sera of naturally EIA infected equids, with levels of reactivity, as well as reference sera available at CRAIE. Different levels of positivity, were obtained by diluting the positive sera in negative serum. The reaction characteristics of each serum, often provided in several replicas in the panel, were verified beforehand by the CRAIE, excluding the presence of aspecific reactions, that was possible in the pools of sera due to the interaction of proteins present in the sera of different donors[2,3].

The panels distributed over the years were made up with a variable number of positive and negative sera (Table 1) but, as recommended by the OIE[3], they always included at least three sera with a reactivity clearly attributable to one of the following categories: strong positive (SP), weak positive (WP) and negative (N).

The expected sera reactivity was set for the AGID as indicated in the SOP-EO-0101.02 Agar Gel Immunodiffusion (Coggins) Test for Equine Infectious Anemia[10] (Fig. 1) and for the ELISAs in view of the outcome obtained by the several commercially available assays.

Before distribution, the sera were subjected to homogeneity and stability tests[3,12], to guarantee the repeatability and uniformity of the expected outcomes.

In recent years, the diagnostic activities carried out at the CRAIE, highlighted an increase in the frequency of weak positive sera, thus since 2012 the IT panels were set up to in-

Table 1 - Composition of the serum panel sent for the equine infectious anaemia interlaboratory comparison in the period 2002-2017.

Year	N° sera	SP	WP	N
2002	13	6	3	4
2003	10	3	5	2
2004	10	5	2	3
2005	12	2	7	3
2006	10	3	4	3
2007	10	5	2	3
2008	10	4	4	2
2009	10	4	3	3
2010	10	5	2	3
2011	10	3	5* (4**)	2* (3**)
2012	30	10	12	8
2013	45	21	9	15
2014	30	6	12	12
2015	30	6	12	12
2017 (AGID OIE)	20	8	4	8
2017 (ELISA)	30	14	7	9

SP: strong positive; WP: weak positive; N: negative.
*Using recombinant p26 as antigen.
**Using the whole EIA virus as an antigen.

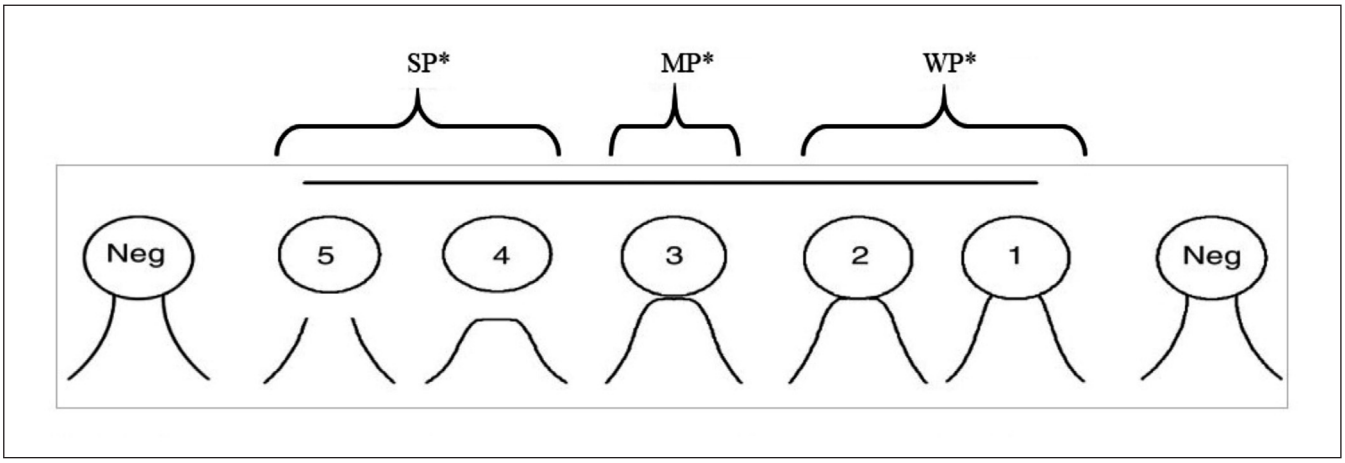

Figure 1 - Examples of positive and negative reactions in the AGID AIE test.
*Positive reactions: SP: strong positive; MP: medium positive; WP: weak positive.
Neg: negative reaction. Modified from SOP-EO-0101.02 Agar Gel Immunodiffusion (Coggins) Test for Equine Infectious Anemia, USDA.

Table 2 - K rating according to Landis and Koch[13].

Kappa statistic	Strenght of Agreement
<0.00	Poor
0.00-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1	Almost perfect

clude a greater number of sera with such reactivity level to obtain a panel that was more representative of the actual field situation and therefore useful for a correct evaluation of the sensitivity of the national laboratory network. From 2012, the year in which the ELISA technique began to be evaluated with the IT, to 2015, the composition of the panels sent for the ELISA and the AGID was identical, while from 2016, when ELISA become the official screening test, a higher number of sera was sent for this method.

The statistical evaluation of the results was carried out using the K statistic proposed by Cohen[13]. The K statistic estimates the agreement between two or more observers who, specifically, make observations according to a nominal rating scale (positive or negative). The use of K allows to standardize the difference between the total agreement observed and the expected concordance due to the case, dividing it by the maximum possible non-random difference. The interpretation proposed by Landis and Koch, shown in Table 2 was used to evaluate the K values obtained from each laboratory and from all the participating laboratories for each of the methods employed[13]. In the statistical elaboration, the positive sera were subdivided into two categories: sera with a strong positive (SP) and a weak positive (WP) reaction; the SP category included also sera with a medium positive reactivity (MP) (Fig. 1).

In the case that the results returned did not comply with the acceptability criteria defined by the CRAIE, the laboratory was asked to provide evidence that it had reviewed the entire analytical process to identify the causes of non-compliance and for these have implemented the appropriate corrective actions. The corrective actions proposed by the CRAIE in the event of a poor to moderate agreement were: to conduct tests under the supervision of CRAIE; or, if the previous action was not possible, repeat the test with a new panel of sera.

In case of a moderate agreement, the CRAIE advised to only the testing of a new panel.

Data analysis

Data of the IT carried out from 2002 to 2017 were considered for this paper. The number of participating laboratories was stratified by year and by technique and the variations over the entire reference period were analysed.

Multiple Kappa value was used to evaluate the overall concordance produced by the laboratory network[14], obtained each year for each technique and the trend of the performance over time was analysed; the average multiple Kappa value was used for a comparative evaluation obtained for the different methods in the entire 2002-2017 period. For laboratories examining the panel with different ELISAs, each set of results was separately evaluated.

From 2006 to 2017, the interval chosen for the greater uniformity of data, the percentage of misclassified sera were assessed, both overall and by reactivity level, for the Coggins test[8] and for the AGID OIE[9]. For the ELISA the error ratio was also evaluated from 2012.

RESULTS

The number of participants for the reference period represented by central and peripheral laboratories of the II.ZZ.SS. and other national and foreign laboratories that specifically requested to perform the IT, are summarized in Table 3. The number of participants underwent a rapid increase from 18 in 2002 to 72 in 2004; this value remained more or less constant in the following years and then sharply decreased after 2012. Since 2013, there was an overall reduction in the number of participating laboratories, with a much more evident decline for the IT on AGID, where membership decreased to reach 23 in 2014 for the Coggins test and 27 in 2017 for the AGID OIE, while the number of adhesions for the ELISA, initially similar to that of the AGID, gradually become predominant and then maintained a more constant trend over time.

The results obtained for the multiple K (Table 4), evaluated as proposed by Landis and Koch were, for the Coggins test, between 0.739 and 0.990, and for the AGID OIE, 0.775-0.990, with a concordance from substantial to almost perfect in both cases; for ELISA, the values of multiple K were between 0.970 and 0.998, indicating an almost perfect agreement. The average multiple K value resulted 0.885 for the Coggins test and 0.926 for the AGID OIE, showing a greater concordance between the laboratories employing the latter technique. For the ELISA, the average multiple K value further increased up to 0.982, with a rise of 5.6% compared to the AGID OIE and 9.7% compared to the Coggins test.

Table 3 - Distribution of laboratories participating to the equine infectious anaemia interlaboratory comparison by technique and by year in the period 2002-2017.

N° Participant laboratories				
Year	Total	Coggins test	AGID OIE	ELISA
2002	18	18		
2003	67	67		
2004	72	72		
2005	67	67		
2006	65	47	37	
2007	68	39	38	
2008	69	39	47	
2009	68	40	43	
2010	69	42	43	
2011	64	35	45	
2012	62	36	40	34
2013	48			48
2014	46	23	28	
2015	38			38
2017	43		27	43

Table 4 - Values of multiple K obtained from the equine infectious anaemia interlaboratory comparisons in the period 2002-2017 stratified by year and technique.

Year	Coggins test		AGID OIE		ELISA	
	N° Lab.	Multiple K	N° Lab.	Multiple K	N° Lab.	Multiple K
2002	18	0.739				
2003	67	0.835				
2004	72	0.960				
2005	67	0.902				
2006	47	0.780	37	0.775		
2007	39	0.940	38	0.975		
2008	39	0.893	47	0.987		
2009	40	0.936	43	0.967		
2010	42	0.950	43	0.983		
2011	35	0.850	45	0.780		
2012	36	0.990	40	0.990	34	0.998
2013					48	0.970
2014	23	0.850	28	0.920		
2015					39	0.980
2017			27	0.960	43	0.980
Mean multiple K		**0.885**		**0.926**		**0.982**

Table 5 - Misclassification percentage on positive sera, as overall and divided by reactivity level (weak and strong) by technique, obtained from the equine infectious anaemia interlaboratory comparisons in the period 2006-2017.

	Overall % Misclassified positives	% Misclassified WP*	% Misclassified SP*
Coggins test	4.40	3.87	0.54
AGID OIE	3.67	3.42	0.25
ELISA**	0.79	0.71	0.08
Total	**8.86**	**8.00**	**0.87**

*WP: weak positive serum; SP: strong positive serum.
**Limited to the period 2012-2017.

Table 5 shows the distribution of error rates by technique, differentiating the strong positive (SP) sera from the weak positive (WP) ones. Of the 14.940 sera tested (4.190 by Coggins test, 5.110 by AGID OIE and 5.640 by ELISA) from 2006 to 2017, the overall error rate was 2.33% (348/14940): of these, 83.91% (292/348) misclassified positive sera and 16.09% (56/348) misclassified negative sera. For positive sera, 90.41% (264/292) of the errors were relative to WP sera. For ELISA, the error rates were much lower than in AGID: the false positives in ELISA represented only 10.27% (30/292). As for the identification of WP sera, from the data obtained in 2012, the year in which an identical panel was distributed for ELISA and AGID, the error percentage for WP sera (Table 6) for the Coggins test was 5.85 times (0.76/0.13) and for the AGID OIE 6.15 times (0.80/0.13) higher than the ELISA. The data of the 2014-2015 period, when the panel distributed for the AGID in 2014 was also used for the ELISA in 2015, presented an error rate for the Coggins test and for the AGID OIE respectively 5.09 (6.52/1.28) and 3.88 (4.96/1.28) times higher than that observed in ELISA (1.28).

Table 6 - Misclassification percentage on weak positive sera using different techniques obtained from the equine infectious anaemia interlaboratory comparisons in the period 2012-2015.

% Misclassified WP*	ELISA	AGID OIE	Coggins test
2012	0.13	0.80	0.76
2014-2015	1.28	4.96	6.52

*WP: weak positive serum.

DISCUSSION

The accreditation of the laboratories and the required participation to the IT with satisfactory results for the monitoring of their quality system immediately determined the increase of participation to the serological EIA IT for of a relevant number of laboratories on national scale (Table 3).
The emanation of the Ministerial Ordinance (M.O.) 14/11/2006[15], reiterated in 2007[16] and in 2010[17], which requested the control of the entire national equid population, helped to keep high the interest of the laboratories to the participation in the IT for the serological methods for the EIA for a substantial number of laboratories.
The increase in the number of samples tested for EIA, according to the legislative provisions, and the need to participate in such circuits with a satisfactory outcome to maintain accreditation, has made the IT an important tool for verifying laboratory performances over the years, and to guarantee for the results.
The decrease in the number of participating laboratories after 2012, coincides with the reduction of controls for EIA at the national level, since the M.O. of 2010[17] was not immediately reiterated and the decision to continue the checks was at the discretion of each region; the reduction

could also be explained by the decrease in the number of laboratories that carry out the diagnosis of EIA as a result of the rationalization policy of activities, adopted by the II.ZZ.SS., for economic and management reasons. The high number of laboratories that have immediately participated in the IT for the ELISA reflects the significant use of this technique in the laboratories, owing to its high sensitivity, fundamental in the screening phase[18]; the implementation of regular controls for EIA since 2007 has made increasingly necessary the use of highly sensitive techniques, able to reveal WP sera, that are difficult to identify as positive in AGID. From a regulatory point of view, the use of ELISA, first alongside the AGID and then in its replacement, was supported by the Ministerial Note Prot. DGSA II/673/P.I. 8d/148 of 12/03/07, which defined the possibility of its employment for the serological diagnosis of EIA in the screening phase, recalling the "Comunicato relativo alle metodologie diagnostiche per le malattie degli equidi riproduttori maschi ai fini della disciplina della riproduzione animale" published in the G.U. n° 66 of 03/21/2005 and the OIE Terrestrial Manual[9]. Subsequently the M.D. 2 February 2016[19] sanctioned the use of the ELISA test for the diagnosis of EIA in the screening phase and of the AGID OIE test[9] exclusively for confirmation and control for international movement purposes. These important regulatory changes determined a further reduction in the number of laboratories that joined the IT for the AGID compared to the ELISA and the use of the AGID method only as reported in the OIE Manual[9].

Regarding the parameters evaluated, the system as a whole presents an acceptable sensitivity for both methods used (ELISA and AGID). However, although the K values obtained are acceptable according to adopted evaluation grid[13], the objective of the control plans to eradicate the infection, would require a value of K close to or equal to 1.

The higher value of multiple K obtained for the AGID OIE compared to the Coggins test, along with greater simplicity in the preparation of the agar plate due to the presence of a single layer and the reduced incubation times (24-48 h instead of 48-72 h) confirms its validity. The high average multiple K value for the ELISA (0.982) shows an almost perfect concordance between the results and a better reproducibility when compared with the AGID, which has lower Kappa values, equal to 0.926 for the AGID OIE and 0.885 for the Coggins test.

The errors in the diagnosis of negative sera could derive from technical errors or transcription of the results, and due to an incorrect understanding of the way in which the results are expressed. From the analysis carried out for the period 2006-2017 (Table 5) it emerges that the errors are mainly relative to WP sera and mainly in the AGID; the explanation could be sought in errors of execution of the test or transcription of the outcomes, but more probably in the sensitivity of the test used[11,18].

The reduced error rate obtained for WP sera using the ELISA (Table 6) derives from the greater sensitivity of the method; a study carried out in 2013 shows the ELISA as able to detect about 20% more positives than AGID[18], therefore more suitable for initial screening during surveillance and useful for recruiting sera eventually undetected in the AGID, to complete the goal of eradicating the infection.

CONCLUSIONS

The IT is an important tool to verify the performance of the diagnostic competence of laboratories and guarantee the results produced and is fundamental for the maintenance of the accreditation[1].

The high sensitivity of the ELISA[18] and the M.D. 2 February 2016[19], which formalized its use for the diagnosis of EIA in the screening phase, justifies the large number of laboratories that immediately adhered to the IT for this technique and the shift of the laboratories towards the use of this technique.

The performances obtained by the laboratories demonstrate the efficiency and stability over time of the EIA serological diagnostic system, which plays a central role in the surveillance and control activities for this infection.

The rapidity of execution and the reduced percentage of error obtained for the WP sera using the ELISA, make this technique a suitable instrument to recruit sera with such reactivity level, often misclassified as negative by AGID, to complete the goal of controlling and eradicating the disease at national level.

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