



NOTE

Virology

Comparison of two agar gel immunodiffusion protocols for diagnosing equine infectious anemia

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ABSTRACT. This study compared agar gel immunodiffusion (AGID) protocols for diagnosing equine infectious anemia. Two commercial testing kits were used: one following the Japanese Act on Domestic Animal Infectious Diseases Control and one following the World Organisation for Animal Health (OIE) manual. From 651 samples tested, both protocols gave identical results for 647 samples (23 samples tested positive; 624 tested negative). Non-specific reactions were observed in 21 samples testing negative by the Japanese protocol, but none were observed with the OIE protocol. The kappa coefficient value was 0.962, indicating almost perfect agreement between the two protocols. This study found no difference in diagnostic agreement between the two protocols, but the OIE protocol produced non-specific reactions less frequently than the Japanese protocol.

KEY WORDS: agar gel immunodiffusion, equine infectious anemia, Japanese act, OIE

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Equine infectious anemia virus (EIAV) belongs to the genus *Lentivirus* in the family *Retroviridae* and causes equine infectious anemia (EIA) in members of the *Equidae* [1]. Horses infected with EIAV manifest as acute, chronic, or subclinical cases showing recurring fever, anemia, depression, edema, hemorrhage, and wasting syndrome. Once infected, all horses remain carriers of EIAV for life, although horses in the chronic phase frequently show no clinical signs.

Transmission of EIAV is mainly by means of contaminated blood carried by blood-feeding insects, and therefore infected horses without clinical signs become potential reservoirs for infecting other horses [1]. The diagnosis and elimination of infected horses is essential to prevent and control EIA because there is no effective therapy for EIA. The diagnosis of EIA can be performed by using virus isolation, molecular methods, and serological methods such as agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA), and immunoblotting [7]. AGID is a standard method for diagnosing EIA and is used worldwide including in Japan. However, there are some minor differences in the AGID protocol as described in the Japanese Act on Domestic Animal Infectious Diseases Control and that described by the World Organisation for Animal Health (OIE) [7]. Concretely, the differences are the concentration of agar and the composition of buffers used to make the agar: 0.8% agar in normal saline solution is used in the Japanese protocol, and 1% agar in borate buffer is used in the OIE protocol. International movement of horses has become common, and because AGID is frequently requested for the diagnosis of EIA when horses move internationally, it is important to confirm whether there are any differences in diagnostic agreement between the two protocols. In this study, we compared the two protocols by using two commercial kits that followed the protocols of the Japanese Act or the OIE.

The kit used for the Japanese protocol was the Purified Antigen of Equine Infectious Anemia Virus for Immunodiffusion Test (Nisseiken, Tokyo, Japan) and the kit used for the OIE protocol was the IDEXX AGID EIA Test (IDEXX Laboratories, Westbrook, ME, U.S.A.). P-337-EFD strain is used as diagnostic antigen in the kit by Nisseiken, whereas the strain in the kit by IDEXX Laboratories is not disclosed. The kit by Nisseiken is licensed for use in Japan. The kit by IDEXX Laboratories follows the OIE protocol and is widely used around the world but is not yet licensed for use in Japan. The Japanese protocol employs 0.8% agar in normal saline solution with 0.1% sodium azide, and the OIE protocol employs 1% agar (Purified agar; Oxoid, Hampshire, U.K.) in 0.145 M borate buffer (9 g H₃BO₃ and 2 g NaOH per litre) with 0.1% sodium azide. Commercial gel (Nippon Bio-Test Laboratories, Saitama, Japan) was used for the Japanese protocol, and the gel for the OIE protocol we made ourselves as described

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above. Serum samples and antigen (50 μ l each) were added to wells cut in the agar plates, and the plates were incubated for 48 hr at room temperature.

Serum samples were collected in 2016 from 562 racehorses and 38 riding horses kept at the Miho and Ritto training centers of the Japan Racing Association, where infected horses have not been detected, and were collected in 2006–2017 from 23 riding horses imported from Belgium and in 2002 from 1 riding horse imported from the United States. In addition, we used 20 retrospective serum samples stored as EIA positive in the laboratory freezer (-20°C) of the Equine Research Institute of Japan Racing Association, which were re-diagnosed as EIA positive by AGID 15 years ago. In 2011, EIA seropositive animals were found among the Misaki ponies [6], which is a breed considered native to Japan and is designated a Japanese National Natural Treasure. Misaki ponies are found only on Cape Toi (*Toi-misaki* in Japanese), Kushima, Miyazaki. This study used seven serum samples collected in 2011 from Misaki ponies infected with EIAV. Totally, 651 serum samples were used in this study.

To analyze the agreement between the two protocols, kappa coefficient values were calculated by using Ekuseru-Toukei 2012 software (Social Survey Research Information, Tokyo, Japan). The kappa coefficient values were evaluated as follows: <0 , poor agreement; 0–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.0, almost perfect agreement [5].

The results of the AGID tests are shown in Table 1. A total of 647 out of the 651 samples were coincident with both protocols, with both protocols showing that 23 samples were positive and 624 were negative. Seven positive samples were collected from Misaki ponies, and 16 positive samples were collected from infected horses and were stored at the Equine Research Institute of Japan Racing Association. Remaining 4 samples that had been diagnosed with EIA previously were negative in this study because the samples should have degraded with age in the freezer. One sample that was positive by the Japanese protocol was inconclusive by the OIE protocol, and vice versa for one other sample. In addition, two samples could not be diagnosed by either protocol. The kappa coefficient value was 0.962, indicating an almost perfect agreement between two protocols. Such inconclusive samples were also observed in the Irish EIA outbreak [3]. These results indicate that it is difficult sometimes to interpret the results of AGID, and in such cases another diagnostic methods such as ELISA and immunoblotting should be used. ELISA tests are more sensitive but less specific than AGID tests [2]. Immunoblotting is not an official test but is sensitive and useful as a supplementary test to reach a consensus when a sample cannot be diagnosed by other diagnostic tests. However, immunoblotting is more laborious and less common than AGID and ELISA tests because the reagents for immunoblotting are currently available only from some specific laboratories [3]. To diagnose inconclusive samples, at least ELISA test needs to be performed in addition to AGID test. Further, considering incubation or seroconversion period, serum samples should be collected from suspected horses again after these horses are isolated. Ideally, inconclusive samples should be diagnosed by a three-tiered strategy, which means that ELISA and AGID tests are conducted, and immunoblotting is finally performed if the result of ELISA is not coincident with that of AGID [2].

Non-specific reactions were observed in 21 of the samples that tested negative by the Japanese protocol; by contrast, no non-specific reactions were observed by the OIE protocol (Fig. 1). All these 21 samples were collected from horses imported from Belgium. Imagawa and Akiyama have reported that mixing horse serum with bovine serum is useful for removing non-specific

Table 1. Comparison of results of two agar gel immunodiffusion protocols for diagnosis of equine infectious anemia according to the Japanese act and according to the OIE manual using 651 serum samples

		Japanese protocol			Total
		+	–	?	
OIE protocol	+	23	0	1	24
	–	0	624	0	624
	?	1	0	2	3
Total		24	624	3	651

+: positive, –: negative, ?: inconclusive.

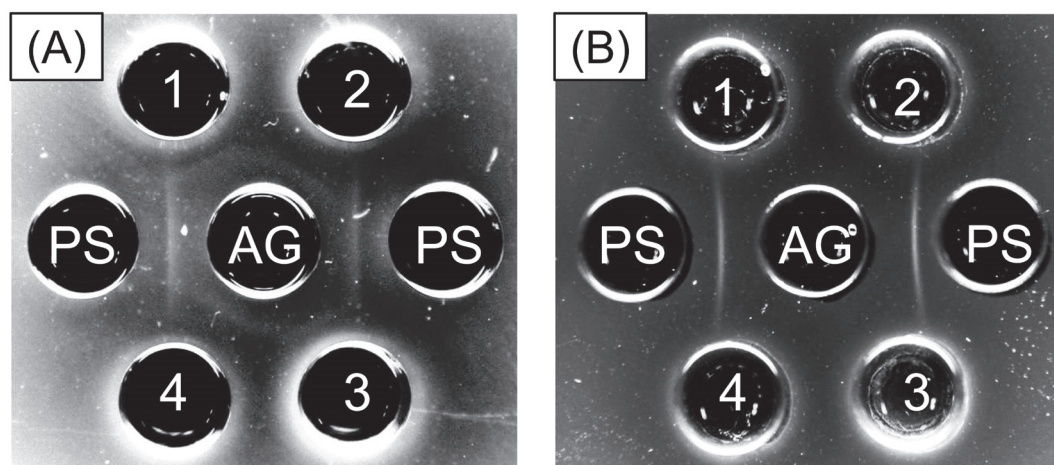


Fig. 1. Results of agar gel immunodiffusion test in accordance with Japanese (A) and OIE (B) protocols. All four samples were diagnosed as negative for equine infectious anemia. Non-specific reactions were produced (A) or were not produced (B) in 1–4 samples. 1–4: Individual horse serum samples, AG: Antigen, PS: Positive serum sample.

reactions [4]. Therefore, 15 μ l of bovine serum (Gibco, Thermo Fisher Scientific, Auckland, New Zealand) was added to 45 μ l of each horse serum that showed a non-specific reaction, and then 50 μ l of the mixture was added to the wells. As a result, non-specific reactions disappeared in all 21 samples, suggesting that these samples have antibodies against antigens derived from cows and that the Japanese kit contains antigens derived from cows. These horses that showed non-specific reactions might have been inoculated with a vaccine containing residual bovine serum used to propagate a virus. Similarly, fetal equine dermal cells are used to amplify EIAV for the antigen, and therefore bovine serum used to cultivate the cultured cells may remain in the Japanese kit. These results suggest that the OIE protocol is easier than the Japanese protocol to judge results in some cases because of the lower frequency of non-specific reactions. In conclusion, this study shows that there is no difference in diagnostic agreement of EIA between two protocols but that the OIE protocol produces non-specific reactions less frequently than the Japanese protocol.

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