Improving a Complement-fixation Test for Equine Herpesvirus Type-1 by Pretreating Sera with Potassium Periodate to Reduce Non-specific Hemolysis

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Non-specific hemolysis has often been observed during complement-fixation (CF) tests for equine herpesvirus type-1 (EHV-1), even when the sera have virus-specific CF antibodies. This phenomenon has also been reported in CF tests for various infectious diseases of swine. We found that the sera from 22 of 85 field horses (25.9%) showed non-specific hemolysis during conventional CF testing for EHV-1. Because pretreatment of swine sera with potassium periodate (KIO₄) improves the CF test for swine influenza, we applied this method to horse sera. As we expected, horse sera treated with KIO₄ did not show non-specific hemolysis in the EHV-1 CF test, and precise determination of titers was achieved.

Key words: complement-fixation test, EHV-1, KIO₄, non-specific hemolysis

The complement-fixation (CF) test is one of the most convenient serological tests available, because it can be applied to the diagnosis of various kinds of infectious diseases just by changing the antigen. The CF test for equine herpesvirus type-1 (EHV-1) is essential in epidemiological studies of abortions, neurological disorders, and pyretic outbreaks on farms and in training centers [6, 11]. However, we have often found it difficult to determine the antibody titers in sera owing to non-specific hemolysis during CF testing. Generally, in the presence of virus-specific antibodies, hemolysis of sensitized red blood cells (RBCs) is inhibited. However, in some sera, inhibition of hemolysis is incomplete, even though virus-specific antibodies appear to be present. Non-specific hemolysis during CF testing has also been reported in swine influenza and other infectious diseases of swine [1–3, 8]. In these studies, one factor in swine sera that enhances the hemolysis caused by guinea pig complement was identified and named as procomplementary factor (PCF). PCF is present in 89.5% of swine sera and is not inactivated by heating for 30 min at 56°C, the conditions under which CF testing is generally performed [2, 5]. PCF activity of PCF is effectively diminished by treatment of the sera with 0.01 M potassium periodate (KIO₄) for 1 hr at 37°C, and this method has been employed in CF tests for swine influenza and swine enzootic pneumonia [1, 8]. Here, we examined sera from horses in the field using a conventional CF test for EHV-1 to determine the incidence of non-specific hemolysis. We then attempted to improve the test by pre-treating the sera with KIO₄.

Paired sera were taken from 2- to 5-year-old Thoroughbred horses (n=85) that had been raised at the Ritto and the Miho training centers of the Japan Racing Association. They had suffered pyrexia (≥38.5°C) in the winter of 2011–12, and some of them had been confirmed, by EHV-1- and EHV-4-specific gG-ELISA, to have seroconverted to EHV-1 or EHV-4 (data not shown) [12]. Pre-sera were taken on the day when the horses had developed pyrexia, and post-sera were taken 14 to 28 days later. Conventional CF testing was performed in accordance with the method reported by Sugiura et al. [10]. For the determination of the endpoint, the highest dilution showing complete inhibition of hemolysis was employed instead of 50% hemolysis.

Non-specific hemolysis was observed in the sera of 22 of the 85 horses (25.9%). In most cases, non-specific hemolysis appeared in both the pre- and the post-serum of the same horse. Figure 1 is an example of the appearance of the sera on the plates and the hemolysis scoring used in judging the...
CF titers. The highest dilution showing a score of 4, which represented complete inhibition of hemolysis (Fig. 1A), was taken as the endpoint, and the reciprocal of the endpoint dilution was taken as the CF titer. In the case of normal serum with EHV-1-specific antibodies, at most 2 dilutions over the endpoint showed slight to moderate hemolysis (scores 1 to 3), and the remaining wells scored 0 (Fig. 1B; normal, N). However, in serum 1, which showed non-specific hemolysis, hemolysis scores of 1 to 3 were given to those wells up to at least 3 dilutions over the endpoint (Fig. 1B). Moreover, with serum 2, which also showed non-specific hemolysis, no dilutions received a score of 4, and slight to moderate hemolysis (score 1 or 2) was observed at more than 5 dilutions, starting from 1:4 (Fig. 1B). According to the definition of the CF titers given above, the titer of serum 1 was judged as 8, and that of serum 2 was less than 4 (Fig. 1B). However, in these cases, the sera seemed to have much higher CF titers because their hemolytic patterns were not typical. For practical purposes, we had provisionally used another definition to determine the endpoint of the sera with non-specific hemolysis to obtain titers that were considered to be more accurate. By this definition, the highest dilution that showed score 3, instead of score 4, was employed to determine the endpoint. The titers by this definition of the 22 paired sera are presented in Table 1.

Next, we performed CF testing with sera pre-treated with KIO₄ in accordance with the method reported by Akao et al [1]. Briefly, 100 µl of serum were incubated for 30 min at 56°C. After the addition of 200 µl of 0.01 M KIO₄ in gelatin veronal buffer (GVB), the mixture was incubated for 1 hr at 37°C. Finally, 100 µl of 10% glycerol in GVB were added, and the resulting mixture was used as 1:4 diluted serum. The remaining procedures of the CF test were the same as those used in conventional testing. For the endpoint determination, the highest dilution showing complete inhibition of hemolysis was employed.

The KIO₄-treated sera showed no non-specific hemolysis at all, even in 22 paired sera that had been difficult to judge using conventional CF testing. As generally observed in the normal sera, hemolysis scores of 1 to 3 were observed in wells with, at most, 2 dilutions over the endpoint, and the remaining wells were scored as 0. In all tested samples, the endpoint became clear, and we could determine the titers more easily and precisely. Figure 2 shows typical examples
Improvement of EHV-1 CF Test

In horse 1, both the pre- and post-serum gave titers of less than 4 in the conventional test, although the post-serum had a titer of 4 when the endpoint definition of score 3 was employed. KIO\textsubscript{4}-treatment yielded a titer of 8 in the post-serum, and seroconversion between the paired sera (a more than 4-fold increase) was detected (Fig. 2). In horse 2, both the pre- and post-serum gave titers of less than 4 in the conventional test even when the endpoint of score 3 was employed. However, the difference between the paired sera in the patterns of the RBCs at higher dilutions suggested that the post-serum sample from this horse had increased CF antibody levels. After treatment with KIO\textsubscript{4}, the pre- and post-titers were determined to be 4 and 8, respectively (Fig. 2). In the case of those sera that presented no problems in the conventional test, the titers yielded by the new method were comparable to those of the conventional testing (data not shown).

We compared the titers of 22 paired sera by the conventional test with the endpoint definition of score 3, and the novel method with pre-treatment of sera with KIO\textsubscript{4}.

| Horse | Conv. Score 3 | KIO\textsubscript{4} |
|-------|---------------|---------------------|
|       | Pre | Post | Pre | Post |
| 1     | <4  | 4    | <4  | 8    |
| 2     | <4  | <4   | 4   | 8    |
| 3     | 8   | 16   | 8   | 32   |
| 4     | 4   | 8    | <4  | 8    |
| 5     | 16  | 32   | 16  | 16   |
| 6     | <4  | 64   | 4   | 32   |
| 7     | 4   | 4    | 8   | 16   |
| 8     | 32  | 8    | 32  | 16   |
| 9     | <4  | <4   | 8   | 4    |
| 10    | 32  | 16   | 16  | 8    |
| 11    | <4  | 4    | 8   | 8    |
| 12    | <4  | 4    | 4   | 16   |
| 13    | <4  | 8    | 4   | 16   |
| 14    | 4   | 4    | 4   | 8    |
| 15    | 64  | 4    | 64  | 8    |
| 16    | 8   | <4   | 8   | 4    |
| 17    | 16  | <4   | 8   | 8    |
| 18    | 4   | <4   | 8   | 4    |
| 19    | <4  | 4    | 4   | 8    |
| 20    | 4   | 8    | 8   | 8    |
| 21    | <4  | <4   | <4  | 4    |
| 22    | 8   | 16   | 8   | 32   |

Conv., conventional method; KIO\textsubscript{4}, novel method with pre-treatment of sera with KIO\textsubscript{4}.

Table 1. CF titers of paired sera (n=22), as determined using the conventional method with endpoint definition of score 3, and the novel method with pre-treatment of sera with KIO\textsubscript{4}.

We observed non-specific hemolysis in about a quarter of horses when we used the conventional CF test. These sera did not show hemolysis when they were mixed with sensitized RBCs without complement (data not shown). Therefore, the sera seemed to have some component that enhanced the hemolysis triggered by the complement, as did the PCF in the swine sera. However, we were unable to determine whether or not sera with titers of less than 4 possessed this activity, because hemolysis occurred in all wells owing to the absence of virus-specific antibodies. Therefore, the incidence of procomplementary activity in horse sera might be higher than that found here. Jensen et al. [7] described the relationship between PCF activity in swine sera and the fifth component of the complement. Although we did not determine which component in the horse sera had procomplementary activity, this component might have the same features as swine PCF, because the activity was diminished by treatment with KIO\textsubscript{4}.

Several substances other than KIO\textsubscript{4} were used in earlier studies to eliminate PCF from swine sera. Phenol or formalin treatment and reduction in pH are effective at reducing procomplementary activity [3–5]. However, a slight decrease in CF titers after treatment with phenol or formalin has been reported [1, 9]. Although pH reduction does not affect the titer, this method requires overnight incubation [3]. In contrast, treatment with KIO\textsubscript{4} requires only a 1 hr incubation and does not affect the CF titer [1]. In this regard, KIO\textsubscript{4}-treatment is superior to the other methods, though its effects on horse sera have remained unknown until now.

In conclusion, we found that 25.9% or more of horse sera had procomplementary activity. Pre-treatment of sera with KIO\textsubscript{4} successfully improved the CF test by making the endpoint clear, and we could easily and precisely determine the CF titers. This method is simple and can be employed in diagnostic laboratories.

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