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Magnetic protein microbead-aided indirect fluoroimmunoassay for the determination of canine virus specific antibodies

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ABSTRACT

Rabies, canine distemper, and canine parvovirus are common contagious viral diseases of dogs and many other carnivores, and pose a severe threat to the population dynamics of wild carnivores, as well as endangering carnivore conservation. However, clinical diagnosis of these diseases, especially canine distemper and canine parvovirus, is difficult because of the broad spectrum of symptoms that may be confused with other respiratory and enteric diseases of dogs. The most frequently used and proven techniques for diagnosing viral diseases include the conventional enzyme-linked immunosorbent assay (ELISA), rapid fluorescent focus inhibition test (RFFIT), mouse neutralisation test (MNT), and fluorescent antibody virus neutralization (FAVN) test. However, these methods still have some inherent limitations. In this study, a magnetic protein microbead-aided indirect fluoroimmunoassay was developed to detect canine virus specific antibodies, human rabies immunoglobulin, CDV McAbs, and CPV McAbs. In this assay, an avidin–biotin system was employed to combine magnetic microbeads and virus antigens (rabies virus, canine distemper virus, and canine parvovirus). Quantification of the targeted virus antibodies was analyzed through indirect fluoroimmunoassay using the specific antigen–antibody reaction, as well as their corresponding FITC-labeled detection antibodies (mouse anti-human IgG/FITC conjugate or rabbit anti-dog IgG/FITC conjugate). The results indicated that the fluorescence intensity increased when a higher concentration of the targeted analyte was used, but the control had almost no fluorescence, much like the conventional ELISA. For human rabies immunoglobulin, CDV McAbs, and CPV McAbs, the minimum detectable concentrations were 0.2 IU/mL, 0.3 ng/mL, and 0.5 ng/mL, respectively. All of these results indicate that this assay can be employed to determine the presence of canine virus specific antibodies. In addition, the method devised here can be utilized as a general protocol in other bacterial and viral marker analysis.

1. Introduction

Rabies, canine distemper, and canine parvovirus are highly contagious and potentially fatal diseases of dogs and many other carnivores. Rabies is a zoonotic viral infection of the central nervous system that is caused by the rabies virus (RV), belongs to the family *Rhabdoviridae* and genus *Lyssavirus*, and gives rise to encephalopathy and ultimately death. Nowadays, rabies continues to be a major public problem that affects humans as well as domestic and wild animals, ranking 11th among the infectious diseases that cause the most human deaths globally (Wunner, 1991; Haupt, 1999). According to a World Health Organization estimate, 50,000 people die from rabies every year, the majority of them in the developing countries of Asia and Africa (World Health Organization, 2005). It has the highest case fatality rate of any infectious disease known to man and is considered to be virtually 100% fatal once symptoms are evident (Wilkerson, 2000). Canine distemper, another contagious viral disease in dogs and many other carnivores, is caused by the canine distemper virus (CDV), a *Morbillivirus* in the *Paramyxoviridae* family. It can be transmitted by aerosols or contact with oral, respiratory, ocular fluids, and other virus-containing exudates. Therefore, dense populations of susceptible animals are needed to sustain epidemics (Williams and Barker, 2001). CDV affects species of the order carnivora and seems to have a major impact on those in the wild and in captivity (Montali et al., 1987). Canine parvovirus infection, the third highly contagious and potentially fatal disease, usually causes severe gastroenteritis in juvenile dogs and myocarditis in neonatal puppies. The causative agent, canine parvovirus (CPV), belongs to genus *Parvovirus* of family *Parvoviridae*.

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It was first recognized in 1978, and within a few years, it spread worldwide and caused a panzootic in dogs (Carmichael and Binn, 1981; Parrish, 1990). CPV now appears to be endemic in almost all populations of wild and domesticated dogs.

The contagious nature and the high mortality rates of these viral diseases make it necessary to speed up the diagnostic procedure in order to quarantine infected individuals and start the appropriate treatment early. Although there is no specific therapy for the deadly diseases, quick and reliable detection methods are extremely critical and essential for measuring the fatal viral diseases at very low levels not only to save lives, but also to save time and money. Additionally, contamination of the environment and the potential transmission of these diseases to other susceptible animals can be prevented by early diagnosis and quarantining the infected animals. Accordingly, a sensitive, specific and rapid method is urgently needed to detect even small amounts of viruses.

Immunoassay has been considered a major analytical method in the fields of clinical diagnostics and biochemical studies because of its high specificity, sensitivity, and affinity between antibody and antigen (Darwish et al., 2007; Pulli et al., 2005; Bromberg and Mathies, 2003; Kurosawa et al., 2006; Park et al., 2006). The conventional enzyme-linked immunosorbent assay (ELISA), rapid fluorescent focus inhibition test (RFFIT), mouse neutralisation test (MNT), and fluorescent antibody virus neutralization (FAVN) test are the proven techniques most frequently used in the diagnosis of viral diseases (Waner et al., 2006; Cliquet et al., 2000; Muhamuda et al., 2007; Renukaradhya et al., 2003; Cliquet et al., 2004; Smith et al., 1996; Fitzgerald, 1996; Cliquet et al., 1998). However, these methods require multi-step processing of samples, live virus or animal/cell cultures, as well as considerable time and expense, which makes them unsuitable for determining the protective status following post-exposure prophylaxis under normal field conditions. Therefore, it remains a challenge to find new approaches that improve the rapidity, simplicity, selectivity, and sensitivity of detection of infectious viral diseases.

Recently, polymer magnetic bead-aided immunoassays have attracted a great deal of attention. These polymer magnetic bead-aided immunoassays integrate the analytical ability of magnetic beads with the high specificity and selectivity of antibody–antigen interactions (Wijayawardhana et al., 2000; Boyaci et al., 2005; Dungchai et al., 2007; Lin et al., 2007; Yang et al., 2008). There are several obvious advantages for using magnetic beads, in contrast to microwells that were previously used as a solid support. The potentially high ratio of attainable surface area to sample volume provides more available binding sites for the analyte. Furthermore, the beads can be dispersed homogeneously in solution, which facilitates the analyte binding to the beads by greatly reducing the diffusional distances, simplifying assay preparation and drastically reducing detection time. The magnetism of the beads allows them to be separated easily from the solution during assay preparation procedures with little or no residual magnetization once the field is removed, facilitating the washing steps (Farrell et al., 2004; Dasso et al., 2002). All of these merits indicate a promising future for magnetic microbeads in rapid, high-throughput screening in clinical analysis (van der Voort et al., 2004). Nevertheless, shortcomings such as biodegradability and toxicity limit further development of polymer microspheres for applications in biological analysis, especially for online and point-of-care testing (Haik et al., 2002; Kramer, 1974). In addition, little effort has been devoted to develop new methods for the detection of canine virus specific antibodies using microbead-aided immunoassay.

In this paper, we describe a magnetic protein microbead-aided indirect fluoroimmunoassay for the analysis of canine virus specific antibodies, human rabies immunoglobulin, CDV McAbs, and CPV McAbs. In contrast to the previously mentioned conventional methods for the detection of canine virus specific antibodies, the magnetic bead-aided indirect fluoroimmunoassay provides a convenient means for rapid and sensitive RV, CDV, and CPV specific antibody assays. Also, the magnetic protein microbeads possess excellent biodegradability, non-toxicity, and biocompatibility (Lee et al., 1981; Gupta and Hung, 1989), and can partially mimic conditions in whole serum samples for clinical use (Chatterjee et al., 2001) compared with conventional magnetic polymer microsphere-based protocols. Using this approach, serial serum samples were analyzed containing the canine virus specific antibodies, human rabies immunoglobulin, CDV McAbs, and CPV McAbs. Moreover, conventional ELISA was employed to evaluate the effectiveness of the microbead-aided indirect fluoroimmunoassay in the detection of canine virus infection.

2. Experimental

2.1. Reagents and materials

Magnetic γ-Fe_2O_3 nanoparticles utilized in this study were prepared from magnetite (Fe_3O_4) according to methods proposed elsewhere (Qu et al., 1999; Sun et al., 2004). For more detailed information on their synthesis see Supplementary information. The rabies virus (RV), rabies virus specific antibody, and human rabies immunoglobulin were purchased from the Institute of Biologic Products (Wuhan, China). Canine distemper virus (CDV), canine parvovirus virus (CPV), canine distemper virus specific antibody CDV McAbs, canine parvovirus virus specific antibody CPV McAbs, and canine parainfluenza virus specific antibody CPIV McAbs were delivered from Animal Epidemic Prevention Ltd. (Beijing, China). Mouse anti-human IgG/FITC conjugate and rabbit anti-dog IgG/FITC conjugate were obtained from Boaosen Biotechnology Ltd. (Beijing, China), and they were utilized as the fluoro-labeled detection antibodies. The BCA Protein Assay Kit and EZ-Link-Sulfo-NHS-LC-Biotinylation Kits were purchased from pierce biotechnology (Rockford, IL). Bovine Serum Albumin (BSA), powdered milk, and Tween 20 were purchased from Sigma–Aldrich (St. Louis, MO). Other reagents and chemicals were purchased from local commercial suppliers and were of analytical reagent grade, unless otherwise stated. Deionized (DI) water (Milli-Q, Millipore, Bedford, MA) was used to prepare aqueous solutions.

2.2. Assay principle for the determination of canine virus specific antibodies

The general principle of the protein microbead-aided indirect fluoroimmunoassay for the detection of canine virus specific antibodies (Fig. 1) starts with the preparation of magnetic BSA/γ-Fe_2O_3 microbeads by combining magnetic γ-Fe_2O_3 nanoparticles and BSA. Then, biotinated antigens (RV, CDV, and CPV) of canine virus specific antibodies were respectively grafted onto the protein beads using an avidin–biotin protocol. Subsequently, canine virus specific antibodies bind to the protein microbeads through specific antigen–antibody reactions, and they can then be assayed using fluoro-labeled detection antibodies, mouse anti-human IgG/FITC conjugate (for human rabies immunoglobulin), or rabbit anti-dog IgG/FITC conjugate (for CDV McAbs and CPV McAbs).

2.3. Preparation and modification of magnetic BSA/γ-Fe_2O_3 microbeads

The preparation of magnetic BSA/γ-Fe_2O_3 microbeads and avidin-modified BSA/γ-Fe_2O_3 microbeads followed the previously reported method with minor modifications (Sahin et al., 2002; Wang et al., 2009). Freshly prepared γ-Fe_2O_3 nanoparticles (90.0 mg) were first dispersed in 1.2 mL of DI water and sonicated for 5 min at room temperature. Then, 300.0 mg of BSA and