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ELISA-on-a-chip for on-site, rapid determination of anti-rabies virus antibodies in canine serum

Jin-Woo Jeon[a], Sung-Min Seo[a], Hee-Soo Kim[a], Jin-Sik Oh[b], Youn-Kyoung Oh[b], Gun-Woo Ha[b], Se-Young Hwang[c], Se-Hwan Paek[a,c,*]

[a] Program for Bio-Microsystem Technology, Korea University, 1, 5-ka, Anam-dong, Sungbuk-gu, Seoul 136-701, Republic of Korea
[b] BioNote, Inc., 2-9, Seogu-dong, Hwaseong-city, Gyeonggi-do 445-170, Republic of Korea
[c] Department of Biotechnology and Bioinformatics, Korea University, Jochiwon, Choongnam 339-800, Republic of Korea

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ABSTRACT

Massive pet vaccination has been needed in most countries to control rabies virus dissemination, and the antibody produced in the body must be monitored. To this end, standard analytical methods involving cell cultures infected with the virus were established; however, this analytical approach requires several days under laboratory conditions. To facilitate antibody testing in the field, we developed a rapid immunosensor that can conduct the enzyme-linked immunosorbant assay (ELISA) on a membrane strip-contained chip (ELISA-on-a-chip; EOC) based on a chromatographic approach. The ERA strain virus particles were used as the capture ligand and immobilized on a predetermined site of the membrane. The assay was performed through the sequential addition of the sample and detection antibody labeled with an enzyme. This allowed us to minimize side effects due to the presence of normal antibodies in the serum sample. Total analysis required approximately 20 min until the final colorimetric signal was generated. The analytical results for 40 canine serum samples, 20 positives and 20 negatives, were highly consistent with those obtained using a reference system, i.e., (Platelia™ Rabies II test kit), approved by the World Organization for Animal Health.

1. Introduction

Rabies is caused by a group of neurotropic viruses classified into four serotypes that belong to the *Lyssavirus* genus of the *Rhabdoviridae* family, which have a conical or bullet shape as visualized by electron microscopy [1,2]. The virus is transmitted commonly to human beings and animals via bited or scratched from the infected animal. Viral infection results in almost invariably fatal encephalomyelitis characterized by extraordinary symptoms such as signs of intense anxiety and nervousness, paresthesia in the area of the bite wound, and painful pharyngeal spasms when offered water (hydrophobia) [2]. Convulsive seizures and progressive paralysis also follows infection, and within a few days the patient can fatally die in a coma. The Office international des épizooties (OIE) urges every country to define rabies as a reportable disease [3,4]. Medication may be effective immediately after post-exposure prophylaxis, but no particular medical treatment is available once clinical symptoms are observed [5].

Since the rabies virus can disseminate through infection between pets and wild animals, the disease is difficult to artificially control and could be raged in cattle farms. International travel and moving abroad with pets have also dramatically increased in recent years. To limit the progression of rabies, several countries have required pre- and post-exposure prophylaxis and pet vaccination [4,5]. The virus strain that is used as a vaccine worldwide, referred to as the Challenge Virus Standard (CVS) strain, is thought to have been derived from the American branch of the original Louis Pasteur virus that was isolated from a rabid cow in Paris in 1882 [1]. The Pasteur virus (PV) strain was obtained in 1965 and is probably derived from the original Louis Pasteur virus. Another strain officially approved for use is the Evelyn-Rokitnicki-Abelseth strain (ERA). This strain was derived by attenuating the SAD strain that originated from a rabid dog in the state of Alabama in the United States (1935) [1,6,7]. Eight other viral strains have been used as vaccines and these organisms vary according to different continents and countries [1].

After vaccination, it is crucial to verify that enough antibodies are being produced in the body to neutralize the viral infection. Antibody tests can also be used to investigate the antibody levels in farm cattle and wildlife populations that inhabit rabies outbreak areas. Virus neutralization (VN) tests in cell cultures (i.e., baby hamster kidney (BHK)-21 for fluorescent antibody virus neutralization

* Corresponding author at: 540 Biotechnology Building (Green Campus), Korea University, 1, 5-ka, Anam-dong, Seongbuk-Gu, Seoul 136-701, Republic of Korea. Tel.: +82 2 3290 3438; fax: +82 2 927 2797.
E-mail address: shpaek@korea.ac.kr (S.-H. Paek).

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(FAVN) test and mouse neuroblastoma (MNA) for rapid fluorescent focus inhibition test (RFFIT)) have been adopted and recommended by OIE as a follow-up test after vaccination [3]. The principle of the VN tests is the neutralization in vitro of a constant amount of rabies virus before inoculating cells susceptible to the rabies virus. If the antibody can neutralize the virus then the virus culture would not expand and no fluorescent signal would be observed after incubation with a labeled detection antibody [8]. Although this method has been internationally recognized as the standard, this approach requires a significantly long analytical time for completion (e.g., 48 h) including the virus culture. Alternatively, the use of an enzyme-linked immunosorbent assay (ELISA) with rabies glycoprotein-coated plates has been shown to be as sensitive and specific as the VN test using cells [3,9]. ELISA can be readily multiplexed to analyze several samples at the same time. In addition, this assay only requires 2 h to complete; however, repetitive washing and reaction processes and laboratory equipment are needed for signal detection. Thus, this approach cannot be used in the field such as at a quarantine station in ports where the animal serum samples are furnished.

Rapid tests in the field can be accomplished using an immunochromatographic assay where a membrane strip is used as a solid matrix for the immobilization of the capture antibody and colloidal gold is used as the signal generator [10]. This approach not only provides analytical results within 10–15 min in one step, but also the colorimetric signal can be visualized by the naked eye, thus eliminating the need for additional instrumentation. However, the detection limit of this approach is significantly worse than the detection limit of ELISA, which may cause false negative results. This problem has been overcome through the development of ELISA-on-a-chip (EOC) technology where a gold tracer was substituted by an enzyme [11]. This field deployable version of ELISA is performed through sequential reactions based on the cross flow of antigen-antibody binding and enzymatic signal generation [12]. EOC can produce a colorimetric or chemiluminometric signal that can be quantified using appropriate detectors, and the sensitivity can be enhanced by 20–50 times compared to the conventional rapid test kit [13,14].

In this study, we developed an EOC biosensor system for the detection of antibodies against the rabies virus in canine serum, which can eventually be used for rapid detection at field sites. For the antibody test, the ERA strain virus antigen was selected as the capture ligand and was immobilized on the solid matrix. This strain was chosen because it is the most widely used vaccine in Korea [15]. The analytical procedure was optimized particularly for the selective detection of the antibody specific to the virus, with minimal interference from non-specific antibody populations in the serum sample. A commercial ELISA kit approved by OIE was used as the reference system to assess the analytical performance of the EOC biosensor. Comparing to the previous works, we attempted to attain at least two technology innovations. First, to detect anti-rabies virus antibodies with high sensitivity, a scheme was developed for sequential bindings of the analyte and labeled detection antibody to the capture antibody site for sandwich complex formation. Second, the enzyme substrate solution for signal generation was supplied in an automatic mode, instead of manual pipetting, by furnishing a built-in solution tank as shown below.

2. Materials and methods

2.1. Materials

Casein (sodium salt type, extracted from milk), bovine serum albumin, EDTA, sodium phosphate monobasic, sodium phosphate dibasic, Tween-20, sodium acetate, trizma base, glycine, hydrogen peroxide, and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO). Succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC), dithiotheritol (DTT), N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl), carbodiimide (EDC), and anti-mouse goat IgG were obtained from Pierce (Rockford, IL). Nitrocellulose (NC) membrane (CNPF-SN12, 10 μm pore size) and glass fiber membrane (PT-R5) were supplied by Advanced Microdevices (Ambala Cantt, India). Cellulose membrane (17CHR, chromatography grade) and glass fiber membranes (Ahlstrom 8980) were purchased from Whatman (Kent, United Kingdom). Horseradish peroxidase (HRP), anti-rabies mouse monoclonal antibody, Platelia™ Rabies II test kit, rabies virus glycoprotein peptide, and insoluble TMB for membranes (TMB-M) were obtained from EMD Chemicals (Gibbstown, NJ), Fitzgerald (Concord, MA), Bio-Rad (Richmond, CA), Anaspec (San Jose, CA), and Moss (Pasadena, ML), respectively. Other reagents used in this study were of analytical grade.

2.1.1. Anti-canine mouse antibody

Monoclonal antibodies were produced according to a standard protocol [16]. Briefly, canine antibodies were first purified as immunogens from sera by affinity chromatography on a protein G column. The immunogens were emulsified with an equal volume of complete Freund's adjuvant and injected intraperitoneally (300 μg/dose) into Balb/C mice. The mice were subsequently injected with the same amount of immunogen emulsified with incomplete Fruend's adjuvant three times in a 1 week-interval. One day after an additional injection of the immunogen (100 μg/dose), the splenocytes were withdrawn and fused with a SP2/O myeloma cell in the presence of 50% PEG 1500. Fused hybridoma cells were screened based on HAT selection [17] and mixed cells producing antibodies reactive to canine antibodies were screened by immunoassays using antigen-coated microtiter plates. Positive cells were cloned twice by limiting dilution and a cell clone (clone CG3) producing an antibody specific to the immunogen was finally selected. The hybridoma cells were expanded by cultivation and used to produce the antibody as an ascitic fluid from Balb/C mice. The antibody was purified on a protein G column and the IgG fractions were pooled, concentrated, dialyzed against PBS, and frozen as aliquots until used.

2.1.2. Capture ligands for antibody detection

Three different antigens, i.e., inactivated rabies virus particles, the glycoprotein peptide, and peptide-BSA conjugates, were prepared and used as the capture ligands in the antibody immunoassays. The rabies virus particles were prepared by expanding the virus (ERA vaccine strain) in a baby hamster kidney epithelial cell line (BHK-21, ATCC CCL-10) as described in a previous report [17]. Briefly, the cells were grown in Earle's minimal essential medium supplemented with glutamine and fetal bovine serum, and cultured for 4 d after infection with the rabies virus. After separating the cells, the virus particles (0.8 L) were transferred to a dialysis bag (molecular cut-off: 6000–8000) and concentrated by immersion in a 50% polyethylene glycol 6000 solution (3 L) at 4 °C for 24–36 h. The particles were suspended in 10 mM phosphate buffer, pH 7.2, containing 140 mM NaCl (PBS) and then spun down by centrifugation at 3000 rpm for 20 min. The supernatant was collected and finally dialyzed in PBS.

The peptide-BSA conjugate was chemically synthesized after activating BSA with an activating agent. BSA was first reacted with SMCC in a 25-excess molar ratio at room temperature for 2 h. The excess reagent was then separated by size exclusion chromatography on a Sephadex G-15 gel column. The activated BSA (final 10 mM) was combined with a 10 fold excess of peptide and the mixture was reacted at 4 °C for 9 h. The conjugation was verified