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Evaluation and comparison of three enzyme-linked immunosorbent assay formats for the detection of ricin in milk and serum

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ABSTRACT

When applied to the detection of a specific protein toxin in food or biological fluids in the incidence of a potential contamination, it is crucial that the assay be both sensitive and specific. In order to identify an immunoassay which is sensitive, simple, and accurate for the detection of ricin in milk and serum, three formats of sandwich enzyme-linked immunosorbent assay (ELISA) were compared utilizing the same pair of antibodies. The ELISA using a biotinylated primary detection antibody and streptavidin-linked horseradish peroxidase (HRP) system was shown to be the most sensitive assay with limits of detection (LOD) of 25 pg/mL in phosphate buffered saline (PBS), 50 pg/mL in non-fat milk, mouse serum, and 100 pg/mL in whole milk. The second best was the ELISA using a streptavidinylated primary detection antibody and biotin–HRP system, the LOD for ricin was 100 pg/mL in PBS and milk, and 1 ng/mL in serum. The ELISA using a non-tagged primary detection antibody and HRP-labeled secondary antibody performed the least sensitive among all and the LOD was 1 ng/mL in all matrices tested. Compared with the direct ELISAs (without using the capture antibody), the sandwich ELISAs were 50–500-fold more sensitive in PBS buffer. Estimation of the accuracy of these immunoassays using the Coefficient of Variability (CV) showed that the most sensitive ELISA format also had the lowest inter- (4.28%) and intra-assay CV (2.15%) although the inter- and intra-assay CV for the other two ELISAs were less than 10% and 6%, respectively, well below the maximum acceptable level. To conclude, the ELISA using a biotinylated primary detection antibody and streptavidin–HRP system is the best assay for detection of ricin in PBS, milk and serum among three ELISA formats tested and the application of this assay will be valuable to food safety research and clinical diagnosis.

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1. Introduction

Ricin is a member of the Type II ribosome-inactivating proteins derived from the seeds of the castor plant, *Ricinus communis.* It consists of two polypeptide chains, A and B, with molecular weights of 32 and 34 kDa, respectively. The ricin A chain is a highly active *N*-glycosidase that inhibits protein synthesis, resulting in cell death by inactivating ribosomes (Endo et al., 1988). The ricin B chain is a lectin that binds to glycoproteins or glycolipids on the surface of target cells and helps the ricin enter cells via receptor-mediated endocytosis (Audi et al., 2005). Ricin is one of the most potent toxins known, with an oral median lethal dose (LD_{50}) of 30 mg/kg in mice (He et al., 2010) and 1–20 mg/kg of body weight in human (Audi et al., 2005). Because of its high toxicity and facile preparation from castor bean, ricin is a potential biothreat agent and has been listed as one of the Category B Agents by the Centers for Disease Control and Prevention. The possibility that bioterrorists could attack the U.S. food supply using ricin has become a public concern. Therefore, identification of a sensitive method for rapid detection of ricin in food and biological fluids is urgently needed. A variety of methods have been developed for the detection of ricin in both buffer and complex food matrices. They range from mouse bioassay (Garber 2008) to cell-free and cell-based assay (Halter et al., 2009; He et al., 2008), immuno-PCR (He et al., 2010), mass spectrometry (McGrath et al., 2011), and multiplex detection (Kull et al., 2010; Garber et al., 2010). But most of these methods require special facilities or expensive equipment that is usually not available in common laboratories.

The enzyme-linked immunosorbent assays (ELISAs) are plate-based assays and have been used broadly for the detection and quantification of substances such as peptides, proteins, antibodies and hormones. Though not the most sensitive assay, ELISA provides several important benefits. Notably it requires only small volumes and hence lesser amounts of reagents; it is easy to adapt to 96-well microtiter plates and use with many different

Abbreviations: CV, coefficient of variability; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LOD, limit of detection; PBS, phosphate buffered saline

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detection systems. Therefore, it has become a common technique for basic research and high throughput applications; it can immobilize reactants to the microplate surface, which facilitates separation of bound from non-bound material during the assay. The ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes within complex matrices. Furthermore, all reagents and equipment needed by ELISA are available in most laboratories. ELISAs can be performed with a number of modifications to the basic procedure. The key step, immobilization of the antigen, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (labeled primary antibody) or indirectly (labeled secondary antibody). The most powerful ELISA is the sandwich assay. In this type of assay, the antigen of interest is bound between two primary antibodies, the capture antibody and the detection antibody. This method is popular because it is more sensitive and robust compared to assays directly immobilizing antigen to the plate. A number of sandwich ELISA formats have been developed for the detection of ricin (Brandon 2011; Guglielmo-Viret et al., 2007), but there is no literature reporting the performance of ELISA in various plate formats through direct comparison.

In this study we aimed to identify and validate a simple, sensitive and repeatable ELISA for the detection of ricin in milk and serum. We evaluated and compared three ELISA formats. The capture antibody in three assays was not modified, but the primary detection antibody was modified in different forms (Fig. 1): ELISA-1. Using a matched set of unlabeled primary detection antibody and HRP-conjugated secondary antibodies; ELISA-2. Utilizing a biotinylated primary detection antibody and HRP–Streptavidin complex for signal amplification; ELISA-3. Utilizing a streptavidinylated primary detection antibody and HRP–biotin complex for signal amplification. The same pair of capture and detection antibodies was used in all ELISA formats to ensure that the observed performance differences were purely due to detection procedure, not properties of the antibodies. All three ELISA formats were optimized thoroughly before being used in this study.

2. Experimental procedures

2.1. Ricin and antibodies used

Ricin and goat anti-ricin polyclonal antibody (pAb) were purchased from Vector Laboratories (Burlingame, CA). Monoclonal mouse antibody, mAb1642 (against ricin A chain), was kindly provided by David Brandon (USDA-ARS-WRRC) (Brandon et al., 2009). Streptavidin–antibody (SA–pAb) conjugation was prepared using the Lightning-Link Streptavidin Conjugation Kit (Innova Biosciences Ltd., Cambridge, UK) following the manufacturer's instructions. Briefly, 100 μl of antibody (1 μg/μl) to be labeled was premixed with 10 μl of LL-Modifier reagent (1 μg/μl) and then added into a vial containing 100 μg of lyophilized L-streptavidin. After incubating the mixture for 3 h at room temperature (RT), 10 μl of LL-quencher reagent was added. The conjugate (SA–pAb) could be used after 30 min or was stored at 4 °C. Biotinylating pAb (biotin–pAb) was performed using LC-NHS-(+)-Biotin (Pierce, Rockford, IL) following the manufacturer's instructions. HRP conjugated biotin and streptavidin were purchased from Invitrogen (Carlsbad, CA).

2.2. Preparation of samples

Non-fat milk and whole milk were purchased from the local grocery store and stored at 4 °C until use. Mouse sera was collected from CD-1 mice (4.5-weeks old, female, 19–20 g) which were obtained from Charles River Laboratories (Hollister, California). The serum was diluted 1:9 in phosphate-buffered saline (PBS: pH 7.3) prior to spiking with ricin. Milk samples were spiked with ricin and tested directly without dilution.

2.3. Direct and sandwich ELISA

For the direct ELISA, a NUNC MaxiSorp microtiter plate (Cat No. 12-565-136 Fisher Scientific) was coated with a set of serial dilutions of pure ricin in PBS, 100 μL/well overnight at 4 °C. The wells were then blocked with 300 μL of 3% BSA in PBS at room temperature (RT) for 1 h. After blocking, the plate was washed 6 times with water and 100 μL/well of the primary detection antibody was added (ELISA-1: add pAb; ELISA-2: add streptavidin–conjugated pAb) and incubated at RT for 1 h. The plate was washed, as above, followed by 1-h incubation with the appropriate secondary HRP-conjugate (ELISA-1: add donkey-anti-goat IgG-HRP; ELISA-2: add biotin-HRP). After washing, 100 μL of Enhanced K-Blue substrate (Neogen Corp., Lexington, KY) was added to each well and incubated at RT for 5 min. The reaction was stopped with 100 μL 0.3 N HCl and the absorbance was measured at 450 nm.

The sandwich ELISA protocol is the same as above, except that the microtiter plates were coated overnight with mAb1642 at a concentration of 4 μg/mL in PBS buffer. Following the blocking and washing steps, serial dilutions of pure ricin in PBS were added and incubated

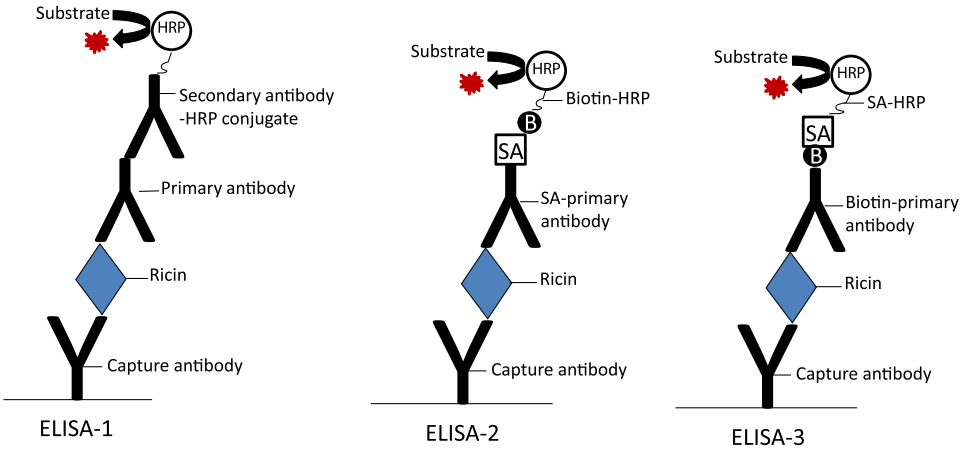

Fig. 1. Schematic representation of the three ELISA formats, depicting the analytical complexes on the surface of an assay well. ELISA-1: Indirect sandwich ELISA using an unlabeled primary detection antibody and HRP-conjugated secondary antibody; ELISA-2: Indirect sandwich ELISA using a streptavidin (SA) tagged primary detection antibody and biotin (B)–HRP; ELISA-3: Indirect sandwich ELISA using a biotin tagged primary detection antibody and SA-HRP.