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

Xiaohua He*, Stephanie McMahon, Reuven Rasooly

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA 94710, USA

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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 immunosassay 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 (LD₅₀) 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 bioterrorism 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.

* Corresponding author. Tel.: +1 510 559 5823; fax: +1 510 559 5768.
E-mail address: Xiaohua.he@ars.usda.gov (X. He).

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ELISA is 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 is 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 i-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

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**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.
at RT for 1 h. Then different detection antibodies and HRP conjugate were used (ELISA-1: non-tagged goat pAb/donkey-anti-goat IgG–HRP; ELISA-2: SA-pAb/biotin–HRP; ELISA-3: biotin–pAb/SA–HRP). To determine the setup that allows for the greatest sensitivity, lowest background, and minimal error, several combinations of antibodies were compared as capturers and detectors. Several dilutions of the secondary HRP-conjugated detection antibody and Bio–HRP and SA–HRP were also tested to determine the optimal concentration. Based on the optimization results obtained from ricin in PBS buffer, we then performed the assays with ricin spiked in milk and serum.

The limit of detection (LOD) is defined as the lowest concentration used for the standard curve at which the average absorbance reading at 450 nm is higher than that of the negative control plus three fold standard deviation. The LOD is matrix, method, and analyte specific. The Coefficient of Variability (CV) is defined as the standard deviation of a set of measurements divided by the mean of the set. The intra-assay CV is a measure of the precision of an assay and reported in this study is an average value calculated from the individual CVs at each known ricin concentration. The % CV for each ricin concentration was calculated by finding the standard deviation of triplicate assays, dividing that by the triplicate mean, and multiplying by 100. It is important to note that the precision of an assay may vary across the assay concentration range.

The inter-assay CV is an expression of day to day consistency. In this experiment, the same set of samples is run in triplicate each day and repeated on three different days to monitor day-to-day variation. The day means for each concentration tested are calculated and then used to calculate the overall mean, standard deviation, and % CV of 3 days. Overall % CV = SD of day means/mean of day means × 100. The average of % CV from all concentrations tested is reported as the inter-assay CV.

### 3. Results

#### 3.1. Evaluation of three ELISA formats for the detection of ricin

The sensitivity of three ELISA formats for the detection of ricin was evaluated by diluting a known amount of ricin sequentially in PBS buffer and determining the LOD (see definition in Materials and Methods). Three replicate measurements were carried out for each serial dilution of ricin (concentration range: 25–100,000 pg/mL) plus an un-spiked negative control. Table 1 indicates that the LOD for ELISA-1 was 1000 pg/mL (at 1000 pg/mL the A450 reading is 0.62, > A450 at 0 pg/mL + 3 × 0.008) and the log-linear range of absorbance signals to the quantity of analytes is between 10,000 and 200,000 pg/mL (R² = 0.97); the LOD for ELISA-2 is 100 pg/mL and the linear range is 100 to 100,000 pg/mL (R² = 0.98); the LOD for ELISA-3 is 25 pg/mL and the linear range is 100 to 5000 pg/mL (R² = 0.99) (Fig. 2).

Direct immobilization of ricin on microtiter plates without using a capture antibody could provide advantages. It is quick because fewer steps are used and it may have low noise because cross-reactivity of capture antibody is eliminated. Therefore, we measured the sensitivity of ELISA-1 and ELISA-2 by direct adsorption of ricin on the plates without using a capture antibody. Table 2 shows that the LOD for both ELISA was 50 ng/mL, which is 50–500-fold higher than those obtained from sandwich assays. There is no significant difference in sensitivity when using monoclonal vs. polyclonal antibody for detection (data not shown). These results suggest that direct capture ELISA is much less sensitive than sandwich ELISA in our experimental conditions. Furthermore, these results were obtained from ricin in PBS buffer, and we believe that it would be less practical for detection of ricin in complex matrices such as milk and serum by direct capture ELISA because these samples contain many other proteins and components that may bind to the microtiter plate surface and thus reduce the sensitivity, and possibly interfere with the assay itself. By coating the plate with a uniform monolayer of mAb and blocking any additional uncoated sites prior to adding the sample, the chance of non-specific binding in the wells by extraneous proteins should be greatly reduced.

#### 3.2. Comparison of the intra- and inter-assay variations among three ELISA formats

In order to compare the precision or repeatability of three ELISA configurations, a set of 10-fold serial dilutions was prepared consisting of four concentrations of ricin (0, 100, 1000, and 10,000 pg/mL) for each of the three ELISA formats. The limit of detection (LOD) was calculated as the lowest concentration where the average absorbance reading at 450 nm for three replicate measurements of ricin spiked in PBS buffer, and we believe that it would be less practical for detection of ricin in complex matrices such as milk and serum by direct capture ELISA because these samples contain many other proteins and components that may bind to the microtiter plate surface and thus reduce the sensitivity, and possibly interfere with the assay itself. By coating the plate with a uniform monolayer of mAb and blocking any additional uncoated sites prior to adding the sample, the chance of non-specific binding in the wells by extraneous proteins should be greatly reduced.
10,000 pg/mL in PBS buffer. On 3 separate days, one set of samples was tested in triplicates and two measures of the Coefficient of Variability (CV): the Intra-Assay CV and the Inter-Assay CV were evaluated. The results are shown in Table 3. The average intra- and inter-assay CVs were respectively 3.47% and 5.00% for ELISA-1, 5.43% and 9.09% for ELISA-2, and 2.15% and 4.28% for ELISA-3 within the concentration range of 0–10,000 pg/mL. It was found that the CVs obtained from all assay formats varied across the assay range, and poorer precision was observed at lower concentrations with the exception of negative controls. Based on the data shown in Table 3, we conclude that the ELISA-3 performed the best. The repeatability of ELISA-1 and ELISA-2 was not as good as that of Format 3, but still at the acceptable level (Intra-assay CVs of less than 10 and Inter-assay CVs of less than 15 are generally acceptable) (McLaren et al., 1981).

3.3. Sensitivity of three ELISA for detection of ricin in milk and serum

To determine the sensitivity of three ELISA methods for detection of ricin in milk and serum, serial dilutions of ricin were spiked into undiluted non-fat and whole milk. For serum, a dilution of 1:9 was made using PBS buffer before ricin was spiked for the purpose of conserving our pool of mouse serum. Table 4 indicates that the LOD for ricin in milk and serum was the same as that in PBS buffer (1 ng/mL) using ELISA-1 method and no matrix effect was observed. The LOD by ELISA-2 was 0.1 ng/mL in PBS buffer and milk (non-fat and whole milk), but the sensitivity was decreased by 10-fold in the presence of serum. The LOD for ricin by ELISA-3 was 25 pg/mL in PBS buffer, 50 pg/mL in non-fat milk and serum, and 100 pg/mL in whole milk. These results suggest that the ELISA-3 was the most sensitive method among three ELISA evaluated although the sensitivity was slightly affected in the presence of milk and serum.

4. Discussion

One of the most important things to measure when evaluating an assay quality is the sensitivity of the assay. This is a measure of the ability to detect very low levels of the antigen of interest. There will sometimes be a trade-off in that the higher the sensitivity of an assay the lower its specificity. It is common practice to apply a highly sensitive technique as a screening test in order to identify problem samples, and then apply more specific tests only to these samples for confirmation. In this study, we selected three standard indirect ELISA formats and evaluated their use for detection of ricin in different liquid matrices. To reflect the true difference in detection technologies, we used the same pair of capture and detection antibodies, blocking, incubation and washing conditions for the three assays. ELISA-1 used an unlabeled primary detection antibody, so the immunoreactivity of the antibody is retained. While the detection antibody in ELISA-2 and ELISA-3 were respectively tagged with streptavidin and biotin, the immunoreactivity of these primary antibodies could be adversely affected. On the other hand, the sensitivity of these ELISA Formats could be increased because each primary antibody has the potential to be conjugated to multiple streptavidin or biotin molecules, allowing for larger signal amplification. Our results indicate that the ELISA-3 reliably detected 25 pg/mL of ricin in PBS, showing a 4-fold improvement in sensitivity over ELISA-2 and a 40-fold improvement over ELISA-1. The high sensitivity observed using the ELISA-3 suggests that the biotinylation of pAb did not affect the immunoreactivity of the antibody. This is reasonable because biotinylation of an antibody increases the molecular weight only minimally, so it may not have an adverse effect on the binding affinity between antigen and antibody. In addition, the use of a long spacer arm between the protein binding site and biotin reduces steric hindrance of the subsequent streptavidin–biotin reaction (Kobayashi et al., 1994). Compared with the ELISA-3, the ELISA-2 was 4-fold less sensitive. This may be due to the large molecular weight of streptavidin. Streptavidin is 60-kD, while biotin is only 244-D. The conjugation of streptavidin to pAb results in a molecular weight 40% greater than that of native antibodies, which could affect the binding affinity (Sung et al., 1995). In this study, we consistently noticed that the ELISA-3 had 3–4-fold higher background noise than the ELISA-2, suggesting the sensitivity of the assay could be increased further with additional effort to reduce the ELISA-3 background noise. The ELISA-1 was least sensitive among the three methods and this low sensitivity was closely associated with a high background noise, suggesting cross-reactivity between the secondary antibody and the capture antibody may occur although the capture and primary detecting antibodies were made in different animal species. In contrast, the use of the streptavidin-biotin amplification systems in ELISA-2 and ELISA-3 led to a significant reduction in background noise and improvements in detection sensitivity. This may be due to the strong interaction between streptavidin and biotin, known to have a dissociation constant $K_d$ of about $10^{-15}$ (Green, 1963). Although both ELISA-2 and ELISA-3 are clearly more sensitive than the ELISA-1, ELISA-2 had a lower reproducibility rate than the ELISA-1 and ELISA-3. This was reflected on the CVs, the ELISA-2’s intra- and inter-assay CVs were 5.43 and 9.09, more than double the amount of CVs generated from the ELISA-3. This may be explained in two ways: 1. Conjugation of antibody to streptavidin may cause steric hindrance to the binding of antibodies, resulting in the increase of intra-day and inter-day variability; 2. Streptavidin is made up of 4 subunits, only one of which is presumed to attach to the antibody, reactivity of unbound subunits to biotin may be inconsistent in each experiment.

The sensitivity of the ELISA-1 and ELISA-2 did not appear to be affected by most sample matrices, with the exception of the
serum (LOD increased 10-fold). However, the detection sensitivity of the ELISA-3 decreased 2-fold in non-fat milk and serum, and 4-fold in whole milk compared to the PBS buffer. This is probably due to the presence of multiple proteins and fat in these matrices affecting the antigen–antibody binding, especially when the antigen level was very low. But overall, the sensitivity of the ELISA-3 in the various matrices tested was still better than the other two ELISA Formats.

In this study, we compared three indirect sandwich ELISA methods for the detection of ricin. The comparison was rigorous and all assays were fully optimized and used the same pair of antibodies and assay conditions for unbiased comparison. The ELISA-3, using the biotinylated primary detection antibody and SA–HRP system was shown to be the most sensitive and reproducible method for detection of ricin in all matrices tested among the three ELISA assays, and the LOD was well below the human oral lethal dose. As with the other two ELISA, this assay can be performed in about 3 h using pre-coated plates, requires little specialized equipment, and can be applied to a high-throughput screening test. Our lab has successfully confirmed the same result for the detection of shiga-like toxins using these three ELISA formats. This study will give insights and direction to the detection of other toxins such as botulinum toxins, cholera toxin and staphylococcus enterotoxin B. The data obtained here validated the use of the sandwich ELISA in the detection of ricin in mouse sera, providing a framework for future diagnostic use of this assay in real human biological samples and ricin poisoning.

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