Defence Science Journal, Vol. 54, No. 1, January 2004, pp. 57-63


# Rapid Detection of Ricin by Sensitising Carboxylated Latex Particles by Ricin Antibodies

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**ABSTRACT**

Ricin is a highly toxic glycoprotein of *Ricinus communis* seeds. The toxin was purified and antisera was raised against ricin in rabbit. Polyclonal antibodies were covalently coupled through a water soluble carbodiimide to carboxylated latex particles in various concentrations (800 μg to 3200 μg protein/0.5 ml). Maximum antibodies binding was obtained at 2400 μg to 3200 μg protein/0.5 ml of 2 per cent (wt/vol) latex particles with a sensitivity of 200 μg toxin per test (9 μg/ml). The sensitivity of latex agglutination test increased as amount of protein bound to the latex particles increased. The optimum sensitivity of test was recorded when latex particles were sensitised with 2800 μg protein/0.5 ml of latex particles. The reagents were stable for one year without loss of its sensitivity. Developed latex agglutination test is rapid, sensitive, and also does not require trained personnel and costly equipment.

**Keywords:** Ricin, sensitised latex particles, immunisation protocol, immunogenic glycoprotein, toxin, toxic lectin, latex agglutination test, protein inhibitor, detection, enzyme-linked immunosorbent assay, ELISA, optic-based biosensors, ricin detection, radioimmunoassay, RIA

## 1. INTRODUCTION

Ricin is a toxic lectin present in the seeds of *Ricinus communis*, commonly known as castor plant. Ricin is highly immunogenic glycoprotein. The molecular weight of the toxin varies from 60 kDa to 65 kDa. Ricin consists of two peptide chains (A and B) held together by a disulfide bond[1]. The A chain possess *N*-glycosides activity and contains the physiologically active site of the molecule. The B chain is a galactose specific lectin and is essential for the binding of the toxin to the cell surface and helps in the entry of ricin molecule into the cell[1]. Ricin inhibits protein synthesis by inactivating ribosomes of eukaryotic cells, leading to cell death[2].

The detection of ricin has been an important area of research, with different methods used for different objectives. A competitive radioimmunoassay[3] that could detect ricin in blood at a concentration 50-100 pg/ml is described. The usefulness of assay was demonstrated with the blood from the patient undergoing treatment with ricin for a tumor. Use of first sandwich enzyme-linked immunosorbent assay (ELISA) with a detection limit of 10 f moles (4 μg/ml) for ricin in buffer using rabbit anti-ricin antibody (reduced IgG and Fab' fragments) complexed with β-D galactose is reported[4]. Subsequent ELISAs were reported for ricin determination in plasma and tissue extracts[5]. Immunocytochemical detection of ricin using an

Revised 19 February 2003

immunoperoxidase method on tissues after ricin injection is also described[6]. Reports[7] are available to improve sensitivity using chemiluminesence as an indicator instead of enzyme substrate used in conventional ELISA.

Recently, fibre optic-based biosensor for the detection of ricin is reported[8]. A sandwich immunoassay has been used to detect ricin. The linear dynamic range of detection for ricin in buffer using the avidin-biotin chemistry is 100 µg/ml to 250 µg/ml. Detection of multiple toxic agents using a planar array immunosensor is reported. Concentrations as low as 25 µg/ml for ricin, 15 µg/ml of *Yersinia pestis F*1 antigen and 5 µg/ml of *Staphylococcal enterotoxin B* (SEB) could be measured[9].

Radioimmunoassay (RIA) and ELISA are the two most commonly used methodologies for the detection of ricin at low concentration levels. Disadvantages of both these methods limit their use. Radioimmunoassay methods developed to detect ricin are time-consuming as these involve overnight incubation[3]. In addition, there is always a problem of handling and disposal of radioisotopes. ELISA offers similar sensitivity as RIA but requires trained personnel and takes longer time to carry out the assay. Biosensors are also costly and time-consuming. Therefore, these methods are not suitable for rapid detection.

In contrast to global scenario, less work has been done in India on the ricin even though, India is the large producer of castor seeds. In spite of the Chemical Weapons Convention (CWC), ricin can be misused by any country and also by the terrorists due to the wild growth of *Ricinus communis* plant. Hence, a quick detection system is very much required. In the present study, an attempt has been made to develop immuno-based latex agglutination test for the detection of ricin.

## 2. MATERIALS & METHODS

### 2.1 Chemicals

Goat anti-rabbit IgG-HRP conjugate, ortho-phenylenediamine (OPD), latex particles, 1-ethyl-3-(3-dimethylamino propyl) carbodiimids (EDC) were purchased from Sigma Chemicals. Other chemicals used were of analytical grade.

### 2.2 Purification of Ricin

Castor seeds were purchased from the local market and ricin was extracted[10]. Further purification of ricin was done by affinity chromatography on acid-treated Sepharose[11]. Under these conditions, lectins bind to the gel matrix (to galactose residues available on the partially acid-hydrolysed matrix). The matrix-bound proteins were eluted with β-D-galactose. These lectins were then separated on the basis of their size using Bio-Gel A-0.5 (BioRad). The fractions eluted were pooled, concentrated, and used for all experiments. Native and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and non-reduced conditions were performed to assess the purity of ricin.

## 3. PREPARATION OF TOXOID & ASSESSMENT OF RESIDUAL TOXICITY

Ricin was treated with 1 per cent formaldehyde for 4 weeks at 37 °C. Unreacted formaldehyde was removed by desalting on *Sephadex* G-25 before it was used for immunisation. Prepared toxoid was assessed for residual toxicity both *in vitro* and *in vivo*. For *in vitro* toxicity assessment, vero cells grown in 24-well plates under routine culture method at 37 °C in an atmosphere of 95 per cent $O_2$, 5 per cent $CO_2$ in Dulbecco's minimum essential media (DMEM) supplemented with 10 per cent fetal bovine serum (including routine supplements and antibiotics). Cells in duplicate were incubated with three concentrations of ricin (10.0 µg/ml, 1.0 µg/ml and 0.1 µg/ml). Control cells were incubated on growth medium only. After 24 h of incubation, toxicity was assessed using the neutral red viability assay[12]. For *in vivo* toxicity testing, the prepared toxoid was injected in mice with a dose of 50 µg/kg, 100 µg/ml and 150 µg/ml in three groups and control (four animals each). Animals were observed for mortality and change in body weight over a period of 4 weeks.

## 4. IMMUNISATION PROTOCOL

The rabbits were used as animal model for raising the antibodies against ricin. The animals

were immunised with toxoid at the dose of 125 μg/kg body weight through subcutaneous injection and test bleeds were made to assess immune status[13]. Immunisation details are given in immunisation protocol. The antibody titer was determined by *Octerlouny's* double immune diffusion as well as by ELISA[14].

IMMUNISATION PROTOCOL

FIRST INJECTION
(3-WEEK INTERVAL)

↓

SECOND INJECTION-FIRST TEST BLEED FOR ASSESSMENT OF SERUM TITERS
(3-WEEK INTERVAL)

↓

THIRD INJECTION-SECOND TEST BLEED
(3-WEEK INTERVAL)

↓

FOURTH INJECTION-THIRD TEST BLEED
(3-WEEK INTERVAL)

↓

BOOSTER INJECTION
(1-WEEK INTERVAL)

↓

NO FURTHER INJECTION-FOURTH TEST BLEED

## 5. *PREPARATION OF SENSITISED LATEX* PARTICLES

Latex agglutination test was standardised and evaluated for the rapid detection of ricin according to the method of Inzana[15]. Briefly, antibodies raised in rabbit against ricin were coupled to carboxylated latex particles (0.88 mm in diameter). The latex particles (0.5 ml of 2 %) were washed three times in 0.1 M carbonate buffer (p*H* 9.6) and then three times in 0.02 M phosphate buffer (p*H* 7.2) in 1.5 ml microcentrifuge tubes. All washes were done at 14,000 × g for 6 min at room temperature unless otherwise stated. Following final wash, the particles were suspended in 0.625 ml of the phosphate buffer and 0.625 ml of freshly prepared solution of 2 per cent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. *HCl* was added dropwise while the solution was slowly vortexed. The tubes were rotated slowly end-over-end for 4 h at room temperature and washed three times with 0.01 M borate buffer, p*H* 8.0. The sensitised latex particles were resuspended in 1.2 ml of borate buffer and antisera (protein concentration ranging from 800-3200 μg) were added, and incubated overnight at 25 °C by end-over-end rotation. To block nonspecific binding, 50 μl of 0.25 M ethanolamine was added to sensitised latex particles and rotation was continued for 30 min. The sensitised latex particles were centrifuged at 14,000 x g for 10 min. Pellet was resuspended in 1 per cent bovine serum albumin (BSA) in borate buffer and rotated for 30 min. Sensitised latex particles were washed one additional time in 1 per cent borate-BSA buffer and made to a final concentration of 0.1 per cent in latex storage buffer (1 per cent BSA (wt/vol), 5 per cent glycerol (vol/vol) and 0.1 per cent $NaN_3$ (wt/vol) in phosphate buffered saline (PBS) for use.

## 6. LATEX AGGLUTINATION ASSAY

An equal volume of antibody-coated latex beads (10 μl) and different concentrations of toxin (10 μl) were placed on glass slide and mixed using a wooden tooth stick. The plate was rotated manually for 1 min to 3 min. The test results were scored as follows:

(a) 4 + Rapid agglutination (within 10 s) of 100 per cent of latex beads, with formation of ring.

(b) 3 + Agglutination of > 75 per cent of latex beads with some ring formation.

(c) 2 + Agglutination of ≥ 50 per cent of latex beads, with no ring formation.

(d) 1 + Fine particle agglutination usually involving ≤ 25 per cent of latex beads.

(e) For control, equal volume of BSA-borate buffer (10 µl) and latex beads (10 µl) were mixed and slide was rotated as per test and used as negative control for grading purpose.

## 7. RESULTS

Ricin treated with 1per cent formaldehyde for 4 weeks at 37 °C was assayed for its residual toxicity both *in vitro* and *in vivo*. *In vitro* toxicity assay of formaldehyde-treated ricin at 10 µg/ml in vero cells did not reduce viability of cells. However, an equivalent concentration of ricin reduced 98 per cent viability of the cells (data not shown). *In vivo* toxicity assay of formaldehyde-treated ricin in mice at doses of 50 µg/kg, 100 µg/kg, and 150 µg/kg did not show mortality in the treated animals. There was a 16.5 per cent increase in the weight of control animals, while treated animals show an increase of 116.0 per cent, 115.3 per cent, and 114.0 per cent for 50 µg/kg, 100 µg/kg, and 150 µg/kg dose, respectively over a period of 4 weeks. The change in body weight was not significant when compared to control animals.

Rabbits were immunised with toxoid and the immune status wrt antibody production was studied by immunogel diffusion technique using 1 per cent agarose. There was no antibody titers after the first and the second injection. A 1:1 titers was observed after the third injection and 1:4 titers after the fourth injection (Table 1). The titer was not enhanced further by a booster injection. Antisera titers raised against ricin toxoid are presented in Fig. 1. The antibody titer was 1:12,800 by ELISA.

Latex agglutination test using sensitised latex particles was standardised for the detection of ricin. Table 2 shows coating of antisera (in varying protein concentrations) on latex particles and detection limits. The results indicate that coating of 2400-3200 µg protein/ 0.5 ml of 2 per cent (wt/vol) latex particles (0.88 mm diameter) was optimal for obtaining a maximum sensitivity of 200 µg/test. The detection sensitivity of test increased as the amount of protein bound to the latex particles increased. The optimum sensitivity was recorded for 2800 µg protein / 0.5 ml latex particles and it was not increased further by adding extra antibodies. Agglutination of 3+ was observed within 1-3 min. The agglutination can be easily read by

**Table 1. Development of antibodies against toxin after repeated immunisation in rabbits***

| Injections | Titers* |
|---|---|
| First dose | -- |
| Second dose | -- |
| Third dose | 1:1 |
| Fourth dose | 1:4 |
| Booster dose | 1:4 |

* Titers by gel diffusion technique

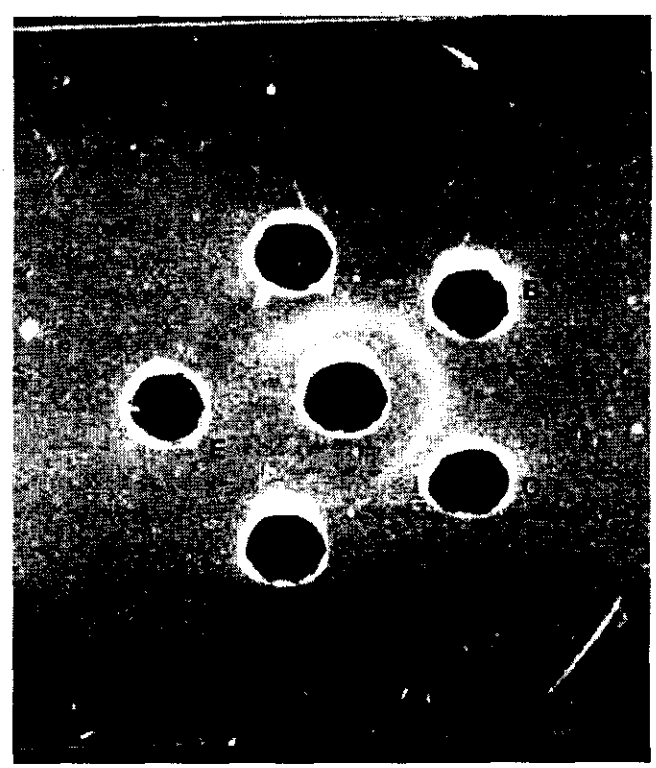

**Figure 1. Immunodifusion of *Ricinus communis agglutinins* and antisera raised against ricin in rabbits. Ricin was loaded in centre well and antisera in surrounding wells. (A): neat antisera, (B): 1:2, (C): 1:4, (D): 1:8, and (E): 1:16 dilutions.**

**Table 2. Coating of antisera (in varying protein concentrations) on latex particles and detection limit**

| Protein coated on latex particles (μg) | Minimum detection limit (μg/ml) | Grading | Observation time (min) |
|---|---|---|---|
| 800 | 300 | 2+ | 5-7 |
| 1200 | 18 | 2+ | 1-3 |
| 1600 | 18 | 2+ | 1-3 |
| 2400 | 9 | 3+ | 1-3 |
| 2800 | 9 | 3+ | 1-3 |
| 3200 | 9 | 3+ | 1-3 |

4+ = 100 % Agglutination, 3+ = 75 % Agglutination

2+ = 50 % Agglutination, 1+ = 25 % Agglutination

nacked eye. The stability study of reagent indicates a self-life of one year without loss in its sensitivity (Table 3). Therefore, adsorption of hyperimmune serum to latex particles was an effective way to prepare sensitive latex particles with optimal sensitivity. Cross reactivity of sensitised latex was evaluated against lipopolysacchride and cyanobacterial toxins. No cross reaction was observed. Therefore, the developed reagent is highly specific for the detection of ricin.

**Table 3. Latex agglutination reagents stability**

| Period (days) | Coated protein on latex particles (μg) | Minimum detection limit (μg/ml) | Grading | Observation time (min) |
|---|---|---|---|---|
| 15 | 2800 | 9 | 3+ | 1-3 |
| 30 | 2800 | 9 | 3+ | 1-3 |
| 60 | 2800 | 9 | 3+ | 1-3 |
| 90 | 2800 | 9 | 3+ | 1-3 |
| 180 | 2800 | 9 | 2+ | 1-3 |
| 360 | 2800 | 9 | 2+ | 1-3 |

4+ = 100 % Agglutination, 3+ = 75 % Agglutination

2+ = 50 % Agglutination, 1+ = 25 % Agglutination

## 8. DISCUSSION

Ricin is considered to be the most toxic substance of plant origin. The $LD_{50}$ of ricin varies from 100 ng to 10 μg/kg body weight (through i.p. route) that depends upon the variety of castor seed, location, and the purity of toxin. Ricin is one of the five most toxic materials known. The other four are: Tetanus toxin, botulinum toxin, diphtheria toxin and gramicidin. One kilogram of ricin is lethal to 3.6 million people, whereas the same quantity of hydrogen cyanide (*HCN*), is lethal to only 16000 people[16]. Just 1 g of ricin is enough to kill 1.5 million guinea pigs.

Mankind has used toxins for homicidal, suicidal, and for malicious mischief. Bulgarian broadcaster Georgi Ivanov Markov was assassinated by ricin-loaded platinum/iridium pellet[17]. Ricin is also included in schedule-I of chemical weapons convention due to its extremely toxic nature. Castor plant is widely distributed and grown in various geographical locations. Same time, isolation of toxin from castor seed is not difficult. Therefore, detection of ricin is important.

In the present study, antibodies against ricin were developed in rabbit with a titers of 1:4 and 1:12800 as determined by gel diffusion and ELISA, respectively. No further increase in titer was observed even after the booster dose. It may be due to the development of immune tolerance. Similarly, serum titers ranged from 800–25,600 using ELISA is reported[13]. The antibody titer also did not increase after the third injection. The viability of cells was reduced to 15.7 per cent wrt control cells, while an equivalent concentration of ricin reduced to 7.0 per cent viability[13].

Agglutination tests using sensitised latex particles are commonly used for the diagnosis of infectious diseases. Antigen detection tests specific for carbohydrate antigens, such as the capsules of *A. pleuropneumoniae, H. influenzae, N. meningitidis* and group-specific carbohydrate antigens of *Streptococcus species* have been reported[18,19]. In the present study, an attempt has been made to develop latex agglutination test for ricin. The optimum coating concentration of antibodies on latex particle was obtained when 2800 μg protein

was allowed to bind in 0.5 ml of 2 per cent (wt/vol) 0.88 μm diameter latex particles. The optimal IgG (purified) binding to carboxylated latex particles was 40 per cent at 800 μg concentration[15]. In this study, protein concentration for optimal binding was more (2800 μg) as whole antisera was used for binding to latex particle. The above developed reagent was stable for 1 year without loss in its binding activity. Stability of reagent was also similar to Inzana's report. The optimum sensitivity to detect ricin in water/buffer was 200 μg/test within 3 min. The other system can detect ricin at a lower concentration, but takes longer time (16 h to 20 h or more) and requires trained personnel as well as costly equipment. The developed test system is rapid, sensitive and does not require trained personnel and costly equipment. The developed reagent has no cross reaction with other toxins.

## ACKNOWLEDGEMENTS

The authors are thankful to Shri K. Sekhar, Director, Defence Research & development Establishment, Gwalior, for providing necessary facilities for this study. Authors are also grateful to Dr R.V. Swamy, Chief Controller (R&D) DRDO HQrs, New Delhi, for his constant encouragement in this study.

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