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Abstract

The purpose of this study was to determine whether rabbit anti-bovine prothrombin/thrombin immunoglobulin Gs (IgGs) would cross-react with bovine factor V/Va-related antigens. Bovine prothrombin, crude thrombin, as well as 2 purified versions of thrombin, that is, thrombin 4A (the previous version of Thrombin-JMI marketed prior to 2008) and 4B (the currently marketed version of Thrombin-JMI), were administered to individual groups of rabbits on days 0, 21, 42, 91, 123, and 151 using standard immunologic methods. Blood was drawn from each rabbit on days 30, 50, 105, 137, and 165 and the pooled antisera from individual groups were purified to obtain the IgGs using protein G affinity columns. By probing bovine factor V/Va samples, the possible cross-reactivity of each IgG collected at different time points (from day 30 to day 165) was explored using Western blotting techniques. The results indicated that rabbit anti-bovine prothrombin and crude thrombin IgGs could cross-react strongly with bovine factor V/Va in an immunization time-dependent manner. However, antibodies generated in thrombin 4A-treated rabbits presented much weaker cross-reactivity with bovine factor V/Va. Furthermore, no cross-reactivity with bovine factor V/Va-related antigens was observed when the anti-bovine thrombin 4B IgG collected at any time point was used. The results suggest that thrombin 4B preparation contains the least bovine factor V/Va contaminants among the bovine prothrombin/thrombin preparations studied and the amount of bovine factor V/Va contaminants in bovine thrombin 4B is too small to elicit the generation of antibodies against bovine factor V/Va in rabbits.

Keywords

bovine thrombin, Western blot, immunoglobulin G, coagulation factor V, topical hemostatic agents

Introduction

Topical bovine thrombin preparations are widely used for surgical hemostasis with an estimated 500,000 patients exposed to these products annually in the United States.^{1,2} Bovine thrombin preparations commonly contain fibrinogen, as well as small amounts of contaminants such as factor V and other plasma proteins. Although topical bovine thrombin preparations have been used successfully in most patients, there are isolated reports of adverse events and deranged hemostasis resulting in severe or refractory bleeding and/or thrombosis.³ Patients exposed to topical thrombin preparations may develop antibodies to bovine thrombin, factor V or various other proteins found in these preparations.⁴⁻⁷ In some cases, these antibodies can, by cross-reacting with the corresponding human coagulation proteins and acting as acquired inhibitors of native human thrombin and factor V, cause a variety of complications ranging from laboratory abnormalities to life-threatening bleeding or thrombotic complications.^{4,8} Many case reports suggest that anti-human factor V antibodies elicited in response to exposure to bovine factor V present in bovine thrombin preparations have played a key role in the development of

coagulopathy.⁹⁻¹² If an antibody-mediated inhibition/depletion of factor V occurs in a patient, that patient may develop a severe and refractory coagulopathy. Unlike reported cases of thrombin inhibitors where bleeding complications are unusual, the majority of the reported patients with factor V inhibitors have had bleeding complications, occasionally fatal.¹³⁻¹⁷

Factor V circulates in plasma as a 330 kd single-chain glycoprotein composed of 6 domains: A1, A2, B, A3, C1, and C2. When activated by thrombin via proteolytic cleavages at arginine residues in positions 709, 1018, and 1545, the B domain is released and a calcium-dependent heterodimer (Factor Va) consisting of a 105 kd heavy chain (A1-A2) and a 71-74 kd light chain (A3-C1-C2) is formed.¹⁸⁻²¹ Factor Va

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then acts as a cofactor for Xa in the presence of calcium ions and a phospholipid membrane surface (the prothrombinase complex), enhancing the rate of prothrombin activation by a factor of 300,000.²² When the primary sequences of human factor V and bovine factor V are compared, there is ~84% to 86% amino acid identity for the corresponding A-type domains and C-type domains, but only 59% for the B domain.²³ The difference in the amino acid sequence of human and bovine factor V/Va may contribute to the development of antibodies to bovine factor V/Va in surgical patients exposed/re-exposed to topical bovine thrombin products contaminated with bovine factor V/Va.

Historically, 3 bovine topical thrombin products (Thrombostat, Thrombogen, and Thrombin-JMI) manufactured by different pharmaceutical companies have been approved by the US Food and Drug Administration (FDA) for the clinical use in the US.²⁴ It has been reported that the purity of these bovine thrombin preparations can differ markedly, with the bovine thrombin content varying from >90% to <30%.^{1,25} Estimated qualitatively, the factor V/Va content in topical bovine thrombin preparations can also vary considerably.^{1,3-8,25-31} Based on the assumption that the contaminants, especially bovine factor V/Va, in bovine thrombin preparations could be responsible for severe adverse reactions in patients, efforts have been made by the manufacturers of bovine thrombin products to reduce or remove factor V/Va from bovine thrombin products to improve the overall safety of the products. In 2008, Terrab et al,³² from King Pharmaceuticals, reported that a newly-adapted membrane-filtration step was highly effective in reducing factor V/Va to an undetectable level. Theoretically, if bovine factor V/Va is completely removed from or reduced to an extremely low level in a topical bovine thrombin preparation, then the application of such product will not elicit the generation of antibodies against bovine factor V/Va. By immunizing rabbits with bovine prothrombin, crude thrombin, and 2 purified versions of thrombin, that is, thrombin 4A (purified by ion-exchange chromatography) and 4B (purified by ion-exchange chromatography plus the reported membrane-filtration step), this study was designed to determine whether generated rabbit anti-bovine prothrombin/thrombin immunoglobulin Gs (IgGs) would cross-react with bovine factor V/Va-related antigens and whether the steps taken in the purification of topical bovine thrombin products would be helpful to reduce the generation of antibodies against bovine factor V/Va.

Materials and Methods

Bovine Prothrombin/Thrombin Preparations

Bovine prothrombin was extracted from freshly collected bovine plasma and then crude bovine thrombin was obtained through the activation of bovine prothrombin by a bovine lung thromboplastin activator. The crude thrombin was initially purified using ion-exchange chromatography to yield an intermediate sample referred to as thrombin 4A. Following elution, the chromatographically purified sample was filtered through

an Omega 100K VR membrane filter, resulting in thrombin 4B.³² The bovine prothrombin, crude thrombin, thrombin 4A, and thrombin 4B preparations used in this study were kindly provided by King Pharmaceuticals (Middleton, Wisconsin).

Bovine Factor V/Va

Bovine factor V/Va was isolated from freshly collected bovine plasma using a modification of the procedure described by Nesheim et al.³³ The obtained protein sample is a mixture of factor V/Va and intermediate activation products as judged by 4% to 20% gradient gels. The bovine factor V/Va used in this study was prepared by Enzyme Research Laboratories (South Bend, Indiana) and serially diluted with saline to concentrations of 500 µg/mL, 250 µg/mL, and 125 µg/mL before loading.

Generation of Anti-Bovine Thrombin Antisera

To generate specific antisera in rabbits, the prothrombin/thrombin preparations were provided to Lampire Biologics (Pipersville, Pennsylvania). Bovine prothrombin, crude thrombin, thrombin 4A, or thrombin 4B conjugated with key-hole lymphocyte hemocyanin (KLH) as a carrier was administered intravenously to individual groups of rabbits at a dosage of 100 µg using standard immunologic methods. Pre-immune blood and antiserum were harvested from each rabbit as shown in Table 1. The antisera obtained from 3 individual rabbits in each treatment group were pooled and stored at -80°C for further IgG isolation.

Immunoglobulin G Isolations

HiTrap Protein G HP columns, 1 mL (GE Healthcare Bio-Science Corp, Piscataway, New Jersey) were employed to isolate IgG from rabbit anti-bovine antisera. The eluted IgG fractions were carefully collected and the concentration of harvested IgGs from each group was adjusted with saline to a 1 mg/mL concentration based on the protein content determined using a modified Lowry's assay.³⁴ All IgGs were aliquotted and stored at -70°C.

Western Blotting

Western blotting was carried out as previously described.^{35,36} In brief, bovine factor V/Va samples were subjected to electrophoresis through 4% to 20% gradient Tris-HEPES-SDS polyacrylamide mini gels and then electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% milk and probed with anti-bovine prothrombin IgG, crude thrombin IgG, thrombin 4A IgG, and thrombin 4B IgG, respectively (2 µg/mL). The membranes were then washed and incubated with horseradish peroxidase conjugated donkey anti-rabbit IgG (1:20,000 dilution). After extensively washing, immunoreactive bands were detected with SuperSignal West Pico Chemiluminescent Substrate followed by film exposure for 1 to 5 minutes.

Table 1. The Generation of Anti-Bovine Thrombin Antisera^a

Day of Schedule	Injection of Antigens	Bleed of Productions
Day 0	1st injection of antigens	Bleed of preimmune serum
Day 21	Second injection of antigens	
Day 30		First bleed of antisera
Day 42	Third injection of antigens	
Day 50		Second bleed of antisera
Day 91	Fourth injection of antigens	
Day 105		Third bleed of antisera
Day 123	Fifth injection of antigens	
Day 137		Fourth bleed of antisera
Day 151	Sixth injection of antigens	
Day 165		Fifth bleed of antisera

^a The antigens used for the generation of antisera include bovine prothrombin, crude thrombin, thrombin 4A, and thrombin 4B. Each of the antigens was injected into 3 individual rabbits at a dosage of 100 µg to elicit the relevant antiserum.

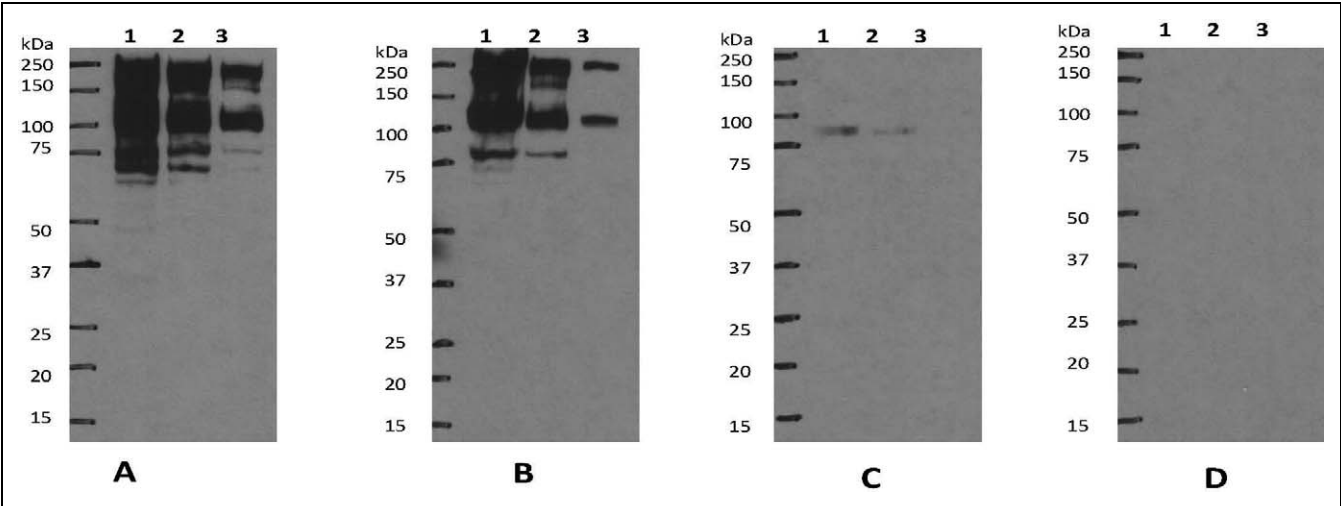


Figure 1. The determination of cross-reactivity with bovine factor V/Va-related antigens using rabbit anti-bovine prothrombin/thrombin immunoglobulin Gs (IgGs) collected 30 days after the first immunization. Serially diluted bovine factor V/Va samples (10 µg, 5 µg and 2.5 µg, lanes 1 through 3) were probed with 2 µg/mL of rabbit anti-bovine prothrombin IgG (panel A), crude thrombin IgG (panel B), thrombin 4A IgG (panel C), and thrombin 4B IgG (panel D), respectively. Horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

Results

Blots identically loaded with serial amounts of bovine factor V/Va sample (10 µg, 5 µg, and 2.5 µg) were probed with rabbit anti-bovine prothrombin IgG, crude thrombin IgG, thrombin 4A IgG, and thrombin 4B IgG collected on day 30 at a concentration of 2 µg/mL (Figure 1). It was demonstrated that rabbit anti-bovine prothrombin IgG as well as crude thrombin IgG exhibited strong cross-reactivity with bovine factor V/Va antigens, revealing multiple immunoreactive bands with molecular weights ranging from around 65 to 250 kd. Rabbit anti-bovine thrombin 4A IgG also cross-reacted with bovine factor V/Va-related antigens although the cross-reactivity was much weaker than those demonstrated by anti-bovine prothrombin IgG/crude thrombin IgG. Compared with the multiple strong immunoreactive bands revealed by

anti-bovine prothrombin IgG/crude thrombin IgG from all 3 amounts of bovine factor V/Va sample loaded, only 1 weaker immunoreactive band at around 90 kd could be detected by bovine anti-thrombin 4A IgG from the higher amounts of bovine factor V/Va sample (5 µg and 10 µg). However, no cross-reactivity with bovine factor V/Va-related antigens was observed when rabbit anti-bovine thrombin 4B IgG was used. Next, the blots loaded with same amounts of serially diluted bovine factor V/Va sample were also probed with the rabbit anti-bovine prothrombin IgG, crude thrombin IgG, thrombin 4A IgG, and thrombin 4B IgG collected on days 50, 105, 137, and 165, respectively (Figures 2-5). Similar to the IgG obtained from day 30, rabbit anti-bovine prothrombin IgG collected from day 50 to day 165 also exhibited strong cross-reactivity with bovine factor V/Va antigens. However, it should

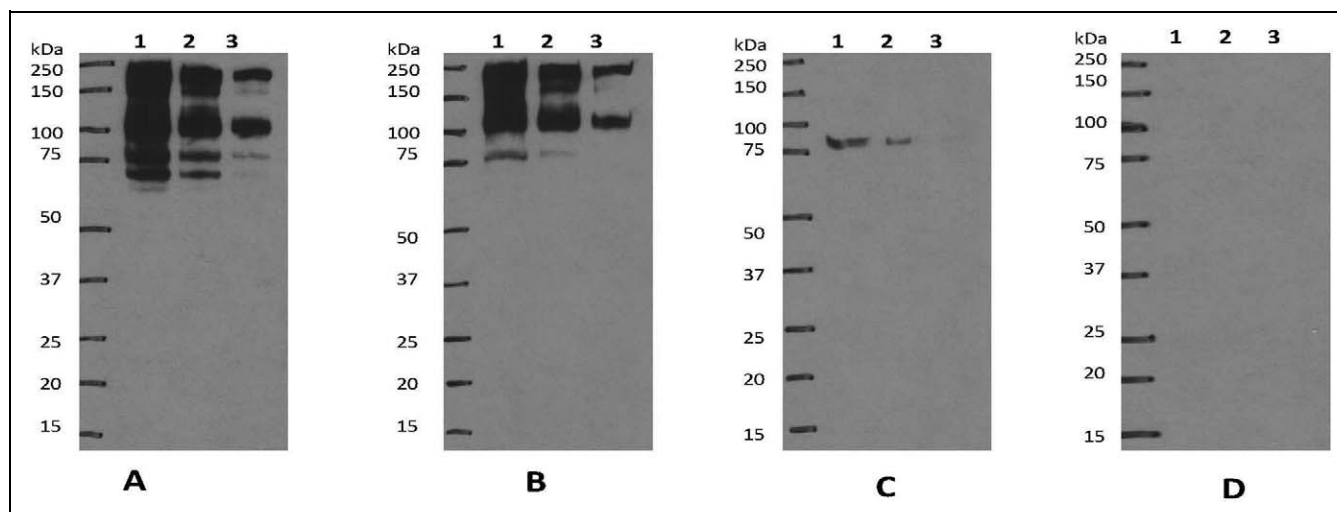


Figure 2. The determination of cross-reactivity with bovine factor V/Va-related antigens using rabbit anti-bovine prothrombin/thrombin immunoglobulin Gs (IgGs) collected 50 days after the first immunization. Serially diluted bovine factor V/Va samples (10 µg, 5 µg and 2.5 µg, lanes 1 through 3) were probed with 2 µg/mL of rabbit anti-bovine prothrombin IgG (panel A), crude thrombin IgG (panel B), thrombin 4A IgG (panel C), and thrombin 4B IgG (panel D), respectively. Horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

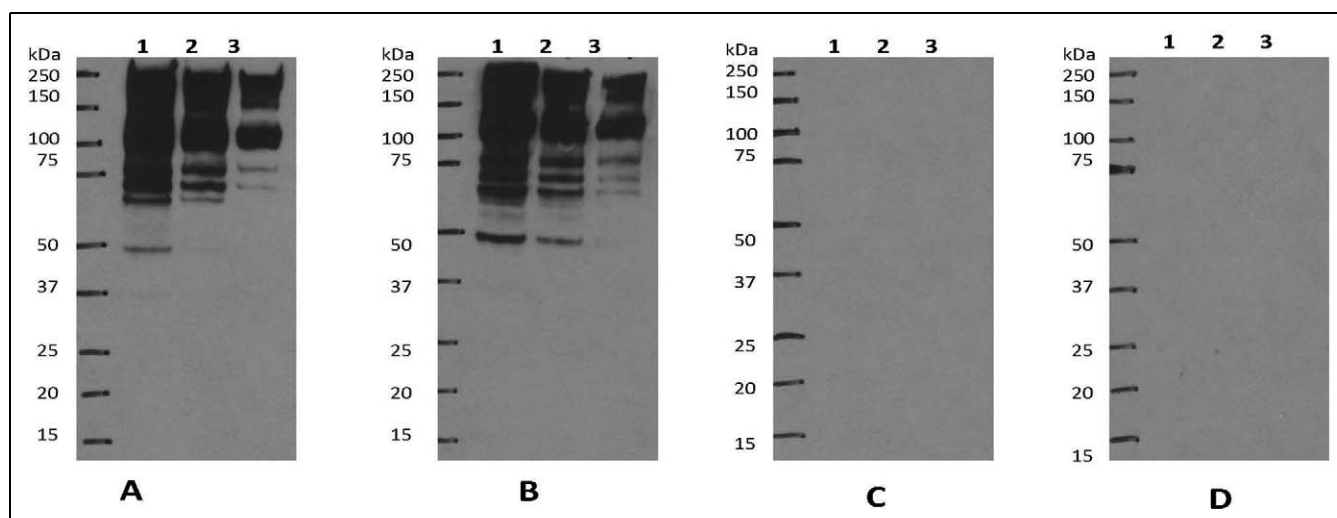


Figure 3. The determination of cross-reactivity with bovine factor V/Va-related antigens using rabbit anti-bovine prothrombin/thrombin immunoglobulin Gs (IgGs) collected 105 days after the first immunization. Serially diluted bovine factor V/Va samples (10 µg, 5 µg and 2.5 µg, lanes 1 through 3) were probed with 2 µg/mL of rabbit anti-bovine prothrombin IgG (panel A), crude thrombin IgG (panel B), thrombin 4A IgG (panel C), and thrombin 4B IgG (panel D), respectively. Horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

be noted that the cross-reactivity with bovine factor V/Va antigens displayed by rabbit anti-bovine prothrombin IgGs from different time points exhibited an immunization-time-dependent manner, that is, the cross-reactivity of the generated IgGs against bovine factor V/Va antigens became progressively stronger (represented by more immunoreactive bands revealed and stronger density of protein bands demonstrated) after multiple injections of bovine prothrombin preparation into rabbits in an extended immunization time period up to 165 days.

As with the results obtained from the IgG collected on day 30, rabbit anti-bovine crude thrombin IgGs collected on day 50 to day 165 also strongly cross-reacted with bovine factor V/Va-related antigens, revealing multiple immunoreactive bands. The number and pattern of immunoreactive bands detected by anti-bovine crude thrombin IgGs from bovine factor V/Va sample were slightly different when compared with those revealed by anti-bovine prothrombin IgG collected at same time point. Moreover, unlike anti-bovine prothrombin

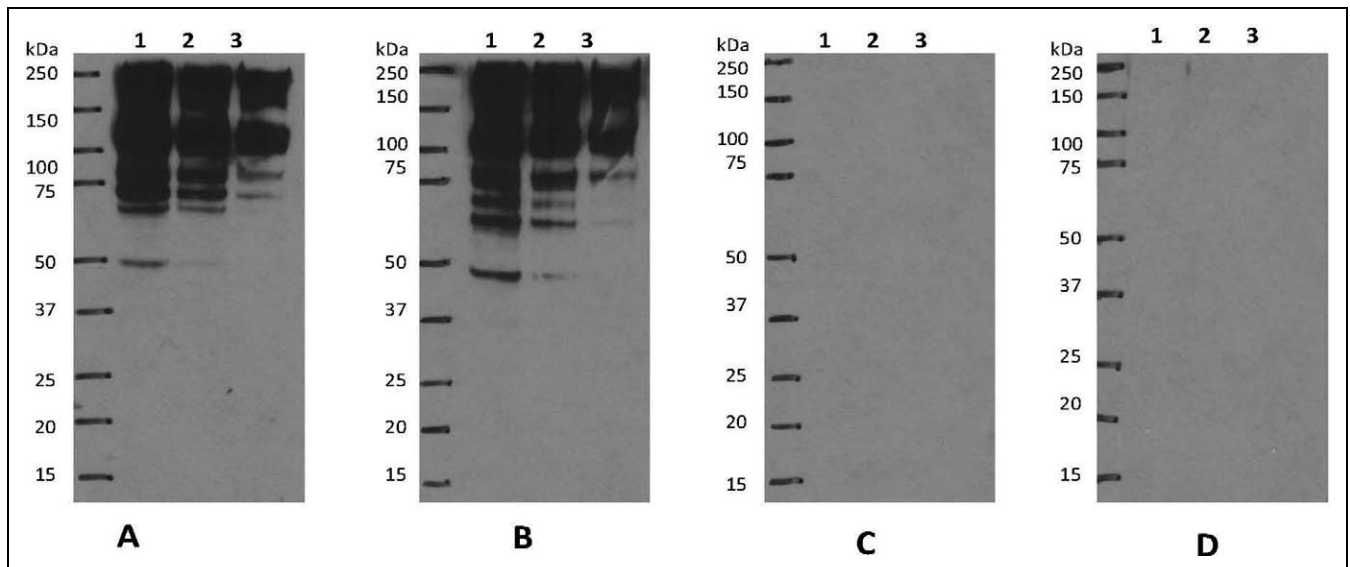


Figure 4. The determination of cross-reactivity with bovine factor V/Va-related antigens using rabbit anti-bovine prothrombin/thrombin immunoglobulin Gs (IgGs) collected 137 days after the first immunization. Serially diluted bovine factor V/Va samples (10 µg, 5 µg and 2.5 µg, lanes 1 through 3) were probed with 2 µg/mL of rabbit anti-bovine prothrombin IgG (panel A), crude thrombin IgG (panel B), thrombin 4A IgG (panel C), and thrombin 4B IgG (panel D), respectively. Horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

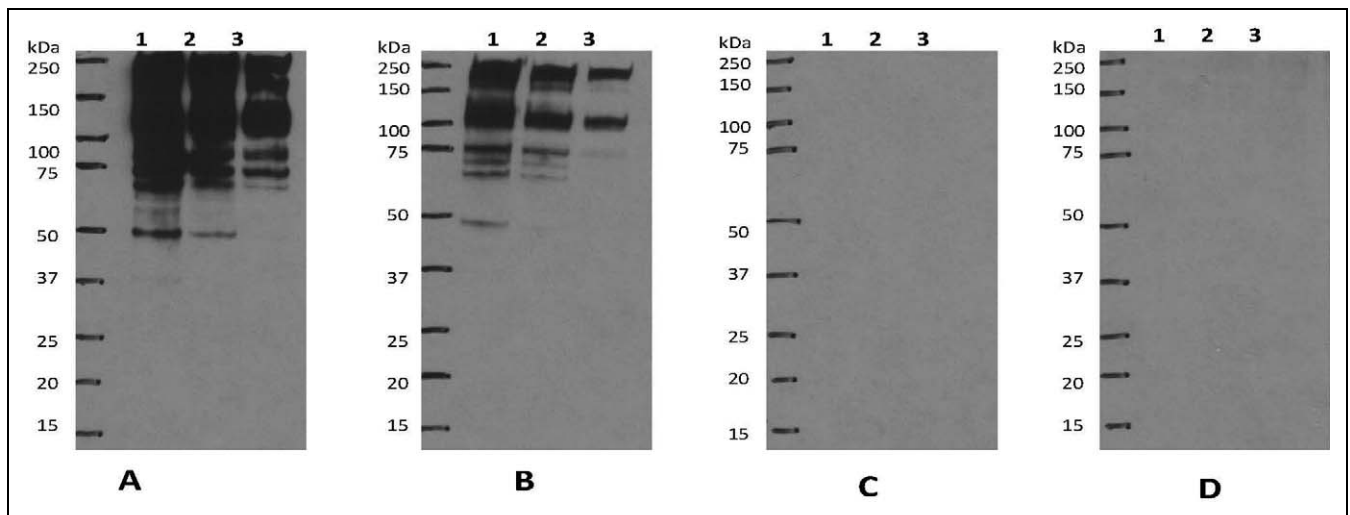


Figure 5. The determination of cross-reactivity with bovine factor V/Va-related antigens using rabbit anti-bovine prothrombin/thrombin IgGs collected 165 days after the first immunization. Serially diluted bovine factor V/Va samples (10 µg, 5 µg and 2.5 µg, lanes 1 through 3) were probed with 2 µg/mL of rabbit anti-bovine prothrombin IgG (panel A), crude thrombin IgG (panel B), thrombin 4A IgG (panel C), and thrombin 4B IgG (panel D), respectively. Horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

IgG in which the titer of the antibody against bovine factor V/Va reached its highest point on day 165, the titer peak of the antibody against bovine factor V/Va in anti-bovine crude thrombin IgG appeared on days 105 and/or 137.

As shown in Figure 2, rabbit anti-bovine thrombin 4A IgG collected on day 50 also cross-reacted with bovine factor V/Va antigens weakly, showing 1 immunoreactive band at around 90 kd in the lanes loaded with 5 and 10 µg of

bovine factor V/Va. When tested at the same concentration of 2 µg/mL, the anti-bovine thrombin 4A IgGs collected on days 105 to 165 did not exhibit any detectable cross-reactivity with bovine factor V/Va-related antigens (Figures 3-5).

As shown with the anti-bovine thrombin 4B IgG collected on day 30, the thrombin 4B IgGs obtained at all other time points did not exhibit visible cross-reactivity with bovine factor V/Va either (Figures 2-5). Cross-reactivity was not observed

even with the concentration of the anti-bovine thrombin 4B IgG collected on day 165 increased to 8 µg/mL (data not shown).

Discussion

In our previous studies,^{35,36} it was found that rabbit anti-bovine thrombin IgGs did not cross-react with bovine factor Va light chain. One possible explanation for the finding is the epitopes that rabbit anti-bovine factor V/Va antibodies can recognize may exist on bovine factor Va heavy chain and/or other activation peptides of factor V but not on bovine factor Va light chain. Moreover, in our previous study, the highest amount of bovine factor Va light chain loaded was 0.2 µg, which may not have been detectable if the titer of anti-bovine factor V/Va antibodies generated in rabbits was very low. In the present study, instead of using bovine factor Va light chain, we have employed a bovine factor V/Va sample that is a mixture of factor V/Va and intermediate activation products. In addition, we have used serial amounts of bovine factor V/Va sample up to 10 µg in the present study to explore the possible cross-reactivity of rabbit anti-bovine prothrombin/thrombin IgGs with bovine factor V/Va.

It was determined from initial screening experiments that when used at a concentration of 2 µg/mL, rabbit anti-bovine crude thrombin IgG exhibited obvious cross-reactivity with bovine factor V/Va and that the minimum amount of bovine factor V/Va sample that anti-bovine crude thrombin IgG could cross-react with was 1.25 µg (data not shown). In addition, it was demonstrated clearly that the most distinctive immunoreactive bands that rabbit anti-bovine prothrombin/crude thrombin IgGs recognized have apparent molecular weights at around 210 kD and 120 kD, respectively. These bands most likely represent the intermediate products of bovine factor V activation.^{18,19} When the amount of bovine factor V/Va sample loaded was increased from 2.5 µg to 10 µg, additional low-molecular weight bands with much weaker immunodensity including the band at 74 kD (bovine factor Va light chain) also became detectable. Therefore, the results obtained from this study were consistent with our previous findings and supported the hypothesis that the rabbit anti-bovine factor V/Va antibodies induced by immunizing rabbits with bovine prothrombin/thrombin preparation mainly bind to intermediate activation products of bovine factor V rather than bovine factor Va light chain and heavy chain.

As mentioned in the Introduction, bovine factor V, a highly immunogenic contaminant in topical bovine thrombin preparations, could be responsible for severe adverse reactions in surgical patients treated with bovine thrombin preparations. It was estimated that up to 50% of patients treated with topical bovine thrombin containing bovine factor V/Va develop anti-bovine factor V/Va antibodies that may also cross-react with and inactivate human factor V/Va.³⁷ It should be noted that due to different purification processes adopted by the various manufacturers of bovine thrombin, bovine factor V/Va content in different topical bovine thrombin preparations can vary widely.^{1,3-8,25-31} From the results of present study, it was

demonstrated clearly that compared with prothrombin and crude thrombin preparations, the bovine thrombin 4A (the previous version of Thrombin-JMI marketed prior to 2008) purified from bovine crude thrombin by the application of ion-exchange chromatography presented much less immunogenicity. The IgGs induced by immunizing rabbits with bovine thrombin 4A preparation exhibited much weaker cross-reactivity with bovine factor V/Va, suggesting that there is much less bovine factor V/Va in the thrombin 4A preparation when compared with bovine prothrombin and crude thrombin preparations. Moreover, no cross-reactivity with bovine factor V/Va was observed when rabbit anti-bovine thrombin 4B IgGs obtained from different immunization-time points were used, suggesting that the newly-adopted membrane-filtration step is highly effective in reducing factor V/Va contaminants in bovine thrombin preparations and the remaining amount of bovine factor V/Va in bovine thrombin 4B preparations is too small to elicit the generation of antibodies against bovine factor V/Va in rabbits.

Conclusion

The results reported in this study indicate clearly that the amount of bovine factor V/Va contaminants in bovine thrombin 4B preparation, if any, is too small to elicit the generation of antibodies against bovine factor V/Va in rabbits. Therefore, our data suggest that the currently used purification steps in the preparation of topical bovine thrombin products are effective in reducing the generation of antibodies against bovine factor V/Va.

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Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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