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Low Cost Alternative Method to Monitor the Electrophoretic Bands Substitution of the Positively Charged Rabbits Seminal Fluids

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Abstract
This paper aimed to evaluate the significance of a non-cost effective double dye technique in visualizing any DNA-seminal proteins interactions. After collecting rabbit's ejaculate and removing sperm cells, ion exchange chromatography was performed to separate seminal proteins on the basis of their charge. Positively charged seminal proteins were eluted, lyophilized, and involved in this study. After incubating this eluted group with a standard reporter DNA, band shift assay was applied on this group. The results were compared. According to this study, we demonstrated the necessity of utilizing a double staining technique for the same band shift gel in order to ensure weather real or false band substitutions were obtained.
Keywords : Rabbits seminal fluids ,DNA , Electrophoresis

1. Introduction

Seminal fluid of mammals contains several barriers that prevent the entry of the exogenous DNA into the sperm cells (Lavitrano et al., 2006). These barriers are identified to explore their inhibitory roles through multiple mechanisms, such as through DNA hydrolytic (DNase) activity (Tanigawa et al., 1975), DNA neutralization activity (Camaioni et al., 1992), or by other mechanisms (Zani et al., 1995). Several papers are described the anti-DNA entry mechanisms that usually available in seminal fluids of several mammals such as mice (Carballada & Esponda, 2001), hamster (Sotolongo et al., 2005), bulls (Tanigawa et al., 1975), and humans (Singer et al., 1983). Thus, it is usually possible to postulate that seminal fluid inhibitory proteins exert their inhibitory role(s) through their binding with the exogenous DNA. Accordingly, several extensive and technically demanded experiments are done in order to demonstrate such mode of interaction such as, Southwestern analysis or through radioactively labeled band shift assay gels (Zani et al., 1995).

In this paper, we try to utilize a simplified tool to deal with seminal proteins – exogenous DNA interaction, in which no sophisticated labeling technique is used. Instead, a highly sensitive and low cost stain is implied to resolve this interaction in such away as to be comparable with several labeling techniques that usually used to identify any band substitution or to demonstrate any change in the mode of seminal fluid after its binding with exogenous DNA.

2.Materials and Methods

2.1 Materials

2.1.1 Kit; PCR SuperMix (Invitrogen – Cat. # 0572-014), PageSilver™ Silver Staining Kit (Fermentas - Cat # K0681), Ladder; DNA size marker; MassRuler™DNA Ladder Mix (Fermentas – Cat. # SM0403), Protein size Marker; Protein low molecular weight size marker (Amersham – code: 17-0446-01), Oligos; 364 bp PCR fragment created from the amplification of GFP gene of gWizGFP vector (Aldevron– Cat. # 5006).

2.1.2 Experimental Animals; Eight New Zeeland sexually mature healthy white rabbits were included in this study. New Zeeland white rabbits were raised in the animal house in the school of bioscience and biotechnology / FST / UKM. They were individually housed under controlled conditions of temperature (19 – 21°C) and standard artificial light (12 hour light and 12 hours dark). A diet of grower rabbits pellets (ad libitum) and fresh water was provided. Animals were cared according to international standards management established for the care and use of laboratory animals in facilities approved by the University Kebangsaan Malaysia Animal Ethics Committee (UKMAEC).

3.Methods

3.1 Semen collection and seminal fluid separation: rabbit's semen was collected by homemade artificial vagina; seminal fluid (supernatant) was separated from sperm cells (pellet) by centrifugation (3000xg for 3 min

each) three times at room temperature. Protein concentration of seminal fluid was measured by UV – visible spectrophotometer (Shimadzu – Japan). Variable concentrations of seminal proteins were incubated with gWizGFP vector at room temperature. Results were analyzed by double stained band-shift PAGE.

3.2 Incubation of rabbit's seminal fluid – 364bpPCR fragment and analysis the incubated mixture by Band shift assay (Lin, 1992**):** 1µg gWizGFP vector was incubated with increasing concentrations of rabbit's seminal fluid (ranging from 1, 2, 3, 4 and 5 µl) and completed to 30µl with sterile deionized water. The incubation was lasted for 60min at room temperature. After incubation, several concentrations were made and analyzed on band-shift polyacrylamide gel according to Lin method (Lin, 1992). Briefly, low molecular weight size marker, gWizGFP vector, as well as gWizGFP DNA – seminal fluid mixtures were applied in 5% band-shift – PAGE, and as the following:

A. Gel preparation: gel dimensions were 10-cm wide by 8-cm long and 0.75-cm thick (Bio-Rad – USA): for each 5ml (5%) band shift PAGE, 60µl (10%) ammonium persulfate and 6µl TEMED was mixed with band shift gel stock solution briefly. Then, the gel solution was applied directly as one continuous phase. Once the gel application was made comb was inserted immediately.

B. Loading the samples: The specimens were mixed briefly, and transferred to the wells of the gel. Each sample was mixed with 4x loading buffer for band-shift assay.

C. Gel electrophoresis conditions: Before undergoing electrophoresis, 200V was applied on the mini-gel for 10 min. Electrophoresis was performed at room temperature using pre-cold electrophoresis buffer at 200V for about 30 minutes.

D. Staining by PageSilver™ Silver Staining Kit: Staining was taken place according to Fermentas instruction manual (cat #K0681).

3.3 Fractionation with ion exchange chromatography (IEC): Seminal fluid proteins were fractionated according to Teixereira *et al.*, 2006 and Villemure *et al.*, 2003 with some modifications.

A. Resin selection: 5 g DEAE cellulose (Sigma Aldrich – USA) was applied as anion exchanger for rabbit seminal fluid fractionation.

B. Regeneration of DEAE-cellulose: All of the preparative steps were conducted in 500ml capacity flask rather than on the glass column. DEAE-cellulose (anion exchanger) was purchased from Sigma. 5 g DEAE-cellulose was slowly added to 300 ml 0.1M sodium hydroxide with gentle stirrer for 30 min (pH reached to 13). The sodium hydroxide solution was discarded and the resin was washed with double distilled water until pH reached to pH 8.0. Then the solution was replaced with 0.1 M hydrochloric acid with gentle stirrer for 30 min (pH reached to 1.0). The resin was washed with double distilled water until pH reached to 3.0. The distilled water was discarded and replaced with 500mM 10X tris buffer pH 8.0 with gentle stirring for 30 min. The 10X buffer was discarded and then the resin was equilibrated with 50 mM tris HCl pH 8.0. After removing more fines, the suspension of DEAE-cellulose resin was transferred into a glass column (2x20 Bio-Rad – USA). Resin –after its packing with 0.01M PBS – was stored in 4ºC until processed.

C. Loading sample: pH of DEAE cellulose effluent was checked up before seminal fluid was applied. Gravity flow was applied instead of peristaltic pump. One ml of seminal fluid containing about 3mg of protein was dissolved in 0.02 M phosphate buffer, pH 7.3, and loaded onto an ion exchange chromatography column (DEAE-cellulose, 2 x 20 cm), which was previously equilibrated with the same buffer.

D. Elution of positively charged proteins: The column was washed and eluted with 0.01M phosphate buffer pH 7.3. The column effluent was collected with manual fractionation, 3ml per fraction. Protein concentrations were spectrophotometrically determined .

3.4 Lyophilization of positively charged rabbit's seminal fluid protein fractions: each five fractions 1 to 5, 6 to 10, 11 to 15, and 16 to 20 were freeze dried (Lyotrap – UK).

3.5 Incubation of eluted and lyophilized positively charged fractions with 364bp PCR fragment and analysis the incubated mixture by band shift gel electrophoresis: Fixed concentration (6µl) 364bp PCR fragment was incubated with different increasing concentrations of DEAE cellulose positively charged fractions (through 1µg, 5µg, and 10µg) for one hour at room temperature. After incubation, each sample – incubated and non-incubated sample –was analyzed on 5% band shift polyacrylamide gel. After electrophoresis band shift polyacrylamide gel was stained with two dyes, the first one was ethidium bromide staining solution (0.625 µg/ml) for 30 min then. The procedure of preparing the band shift gel for staining was taken place by placing the gel – glass plate in an upside down position (the glass plate upward and the gel downward) in ethidium bromide destaining solution on rotary shaker for 1 min. Then, using a sharp polyethylene tip, margins were slightly separated from the glass plate firmly attached gel. Then, the gel was placed again on the rotary shaker and rotated in the same position for additional 1 min. the glass plate was left from one of its ends repeatedly until all the gel was removed from the glass plate. After staining, picture was taken by photodocumentation unit (Alpha Innotech – USA). After staining with ethidium bromide, the same gel was stained by silver staining kit. After finishing with silver staining procedure, gel was place on a white glass plate (Amersham – Sweden) and picture

was taken by a digital camera (Sony – China). Ethidium bromide stained and silver nitrate stained gel pictures were compared.

4.Results and Discussion

4.1 Comparison between 364bps PCR fragment incubated and non-incubated rabbits seminal fluid on single and double stained band shift – PAGE: No more than five micrograms of seminal fluid was incubated with fixed concentrations of PCR fragment (figure 1). This was because the apparent hydrolysis of this fragment. Two activities was identifies in band shift PAGE; the first one the classic DNase activity, and the second was a slight DNA retardation activity.

4.2 Single and double stained Band shift assay PAGE of 364bp PCR fragment of incubated and non-incubated rabbit seminal fluid: Although band shift PAGEs were originally designed to detect electrophoretic band dislocation in nucleic acids only and not in proteins (Ausubel *et al.*, 1989), but the inclusion of double staining technique for the same gel could sometimes considered as an invaluable and helpful tool to give more data could not be provided by a single staining technique. In figure (2) more data were provided by silver nitrate as a second stain for the same gel instead of using any commercially available and cost effective labeling methods (Bayarsaihan *et al.*, 1998, Millership *et al.*, 2004) since the sensitivity of staining with silver nitrate was equal to some staining techniques to the sensitivity of radioactive method (Christensen *et al.*, 1999).

The exact location of shifted band with respect to electrophoresed seminal proteins was made known. In this figure, using only ethidium bromide was sufficient only to observe band substitution without providing further details. While, applying the additional silver nitrate dye identified only the electrophoretic repulsion of the positively charged fractions. By utilizing the second dye it was so easy to see how much repulsive forces were existed on the electrophoresed positively charged seminal fractions. Moreover, the second stain showed that there was proportional relationship between the amount of positively charged seminal fractions and the degree of repulsion noticed on the band-shift PAGEs. Therefore, instead of getting one piece of information of this run, three pieces of information were obtained. This double staining technique could identify DNA retardation extent, the degree of protein repulsive forces, as well as the relationship between DNA retardation and the degree of protein repulsive forces through the gel.

Consequently, when such seminal fluid positively charged fractions were incubated with exogenous DNA, it was mistakenly demonstrated that a sort of band substitution was identified. This was occurred when only DNA binding stain (ethidium bromide) was used. But another conclusion was obtained when both DNA – protein binding stain (silver nitrate) was used. In case of using this second stain, it was demonstrated that the band substitution was just occurred as a result of DNA neutralization only because of the altered nature of these positively charged fraction. Despite the fact the band shift assay was not designed to give high resolution of proteins since it was specific for DNA only, but the necessity of using another dye was so mandatory to confirm the results obtained in the first DNA binding stain was used.

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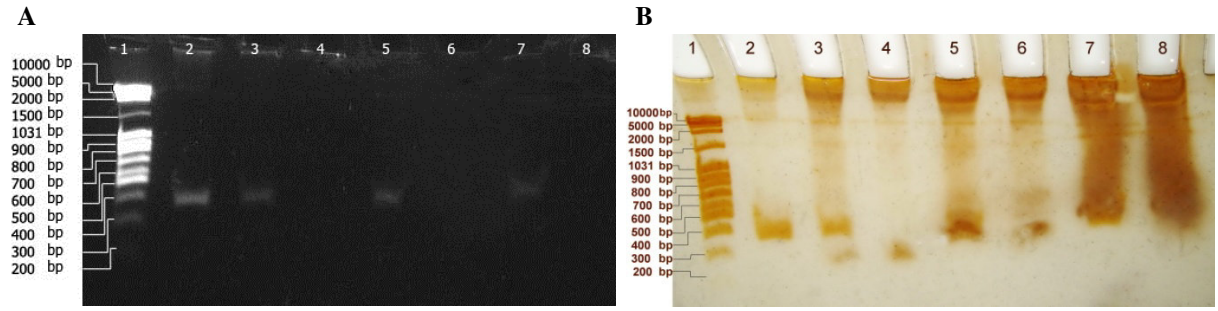

Figure (1): Comparison between 364bps PCR fragment incubated and non-incubated rabbits seminal fluid sample on ethidium bromide stained (A) and silver stained (B) band-shift assay polyacrylamide gel. Lane 1: 15µl DNA size marker (Fermentas). Lane 2: 3 µl 364bps PCR fragment (amplified from gWizGFP vector). Lane 3: 12.5 µl taken from incubation of 3 µl 364bp PCR product with 1 µg rabbit seminal fluid. Lane 4: 12.5 µl taken from 1 µg rabbit seminal fluid. Lane 5: 12.5 µl taken from incubation of 3 µl 364bp PCR product with 3 µg rabbit seminal fluid. Lane 6: 12.5 µl taken from 3 µg rabbit seminal fluid. Lane 7: 12.5 µl taken from incubation of 3 µl 364bp PCR product with 5 µg rabbit seminal fluid. Lane 8: 12.5 µl taken from 5 µg rabbit seminal fluid. bandshift-PAGE electrophoresis conditions: polyacrylamide concentration 5%. Voltage applied: 200 V (15.38 V/cm), run time: 30 min.

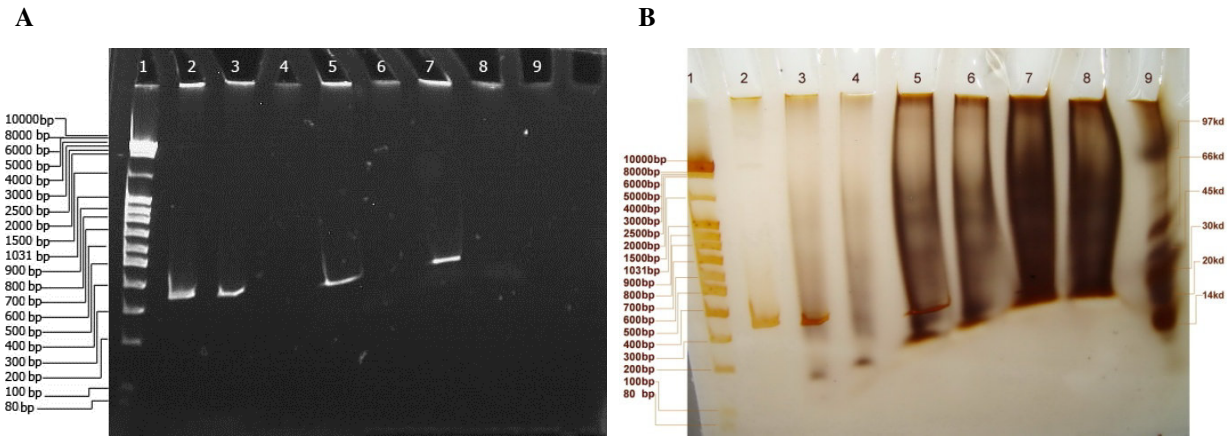

Figure (2): Comparison between 364bps PCR fragment incubated and non-incubated DEAE cellulose separated positively charged fractions on ethidium bromide stained (A) and silver nitrate stained (B) band-shift assay polyacrylamide gel. Lane 1: 15 µl DNA size marker (Fermentas). Lane 2: 6 µl 364bp PCR product. Lane 3: 15 µl taken from incubation of 6 µl 364bp PCR product with 1 µg lyophilized positively charged seminal fractions. Lane 4: 15 µl taken from 1 µg lyophilized positively charged seminal fractions. Lane 5: 15 µl taken from incubation of 6 µl 364bp PCR product with 5 µg lyophilized positively charged seminal fractions. Lane 6: 15 µl taken from 5 µg lyophilized positively charged seminal fractions. Lane 7: 15 µl taken from incubation of 6 µl 364bp PCR product with 10 µg lyophilized positively charged seminal fractions. Lane 8: 15 µl taken from 10 µg lyophilized positively charged seminal fractions. Lane 9: 8µl low molecular weight protein size marker (Amersham). bandshift-PAGE electrophoresis conditions: polyacrylamide concentration 5%. Voltage applied: 200 V (15.38V/cm), run time:30min.