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Characterization of fertility associated sperm proteins in Aseel and Rhode Island Red chicken breeds

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ABSTRACT

This study focused on characterization of fertility associated proteins in Aseel and RIR roosters and was conducted on two generations of birds. Roosters were divided into high (> 50%) and low fertility groups (< 50%) based on sperm function tests and fertility rate in both the generations. Polyclonal antibodies were raised in rabbits against sperm proteins of first generation highly fertile roosters and tested for characterization of fertility associated sperm proteins in the second generation of same roosters. IgG-fraction against proteins (Anti-SP-IgG) was reacted with sperm proteins of both high and low fertile roosters of second generation on immunoblots. Sperm proteins present in highly fertile roosters were further characterized by Mass Spectrometry (MS). Use of SDS-PAGE for evaluation of sperm extracts of Aseel and RIR breeds resulted in resolution of 16 and 10 proteins on 12% acrylamide gels. Anti-SP-IgG reacted with eight and ten sperm proteins of Aseel and RIR roosters on immunoblots. The SDS-PAGE and immunoblotting analysis also indicated a variation in sperm proteins among two breeds and high/low fertile roosters. The MS analysis indicated matching of 20, 30, and 20, 25 kDa proteins (associated with high fertility rate) of Aseel and RIR roosters with immunoglobulin kappa chain variable, phospholipase A_2 (PLA2), hypothetical N332-08551 partial and cystatin like partial proteins with a top score of 41, 46, 52 and 43, respectively. Considering the function and importance of matching proteins in male reproduction, these proteins may be further explored as potential markers for fertility evaluation of Aseel and RIR roosters.

1. Introduction

Artificial insemination in the poultry industry is the key to intensive rearing of breeding roosters. The use of high fertility males is an integral part for artificial insemination. Although the use of AI technology has resulted in many economic benefits, it is highly imperative that high standards for rooster breeding and selection are used for determining semen quality. Semen quality traits are generally overlooked as compared with other identifiable economically important characters in all types of chicken breeds. Achievement of considerable genetic improvement for growth traits in broilers has led to a steady decrease in reproductive efficiency. Hatchability of eggs is influenced by many factors and varies among different breeds (Lariviere et al., 2009; Kingori, 2011). Fertility of roosters also depends on the capacity to mate and quantity and quality of semen (Brillard, 2003; Mavi et al., 2017; Feyisa et al., 2018).

Spermatozoa are highly specialized cells and have specific metabolic pathways compartmentalized in different regions. Unlike

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other cells, spermatozoa are markedly differentiated. Sperm have large variations in genetic, cellular and chromatin structures, which contribute to fertility/infertility, embryo development and heredity. Physiologically normal spermatozoa are required for successful fertilization. Suboptimal sperm quality with abnormal motility, morphology, concentration, DNA fragmentation, chromatin stability and genetic composition has been directly male infertility (Sharlip et al., 2002; Natali and Turek, 2011; Singh and Agarwal, 2011; Park et al., 2012; Muratori et al., 2015). Traditional available methods to detect male fertility in mammals include sperm morphology, motility, cervical mucus penetration assay, acrosome reaction, sperm zona pellucida penetration assay as well as use of some less frequently utilized methods. Semen quality of different breeds of chickens have also been analyzed using sperm variables (motility, count, viability, membrane and acrosome integrity: Hermiz et al., 2016; Mavi et al., 2017). Clinical assessments of the values for these variables and association with fertility are still not widely accepted. Marker proteins of fertility, therefore, could be an appropriate approach to determine male fertility (Rahman et al., 2013).

Proteomic studies on mammalian spermatozoa have revealed the characterization of different proteins in spermatozoa that are responsible for the regulation of normal, defective sperm formation and fertility (Ashrafzadeh et al., 2013). There has been use of proteomic techniques such as SDS-PAGE, 2-D, MS and Difference gel electrophoresis (DIGE) to identify sperm proteins related to fertility in mammals (Dacheux et al., 1998; Amours et al., 2010; Shanmugam et al., 2016). Reports on proteomics of avian spermatozoa, however, are limited. The importance of surface proteins of rooster's sperm traversing the vagina and sperm storage tubules was highlighted by Steele and Wishart (1996). Turkey seminal plasma contains three proteinase inhibitors (Słowinska et al., 2008). When there are normal physiological conditions, these inhibitors form tight, reversible complexes with acrosin (Słowinska et al., 2010). Turkey seminal plasma single domain kazal inhibitors are involved in the control of proacrosin activators in semen (Słowinska et al., 2012).

The present study, therefore was based on the characterization of proteins associated with fertility of Aseel and Rhode Island Red (RIR) roosters using SDS–PAGE, immunoblotting and mass spectrometry.

2. Materials and methods

This study was conducted at Poultry Research Farm, Directorate of Livestock Farms and Reproductive Biology Laboratory, Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India during the period June 2017 to August 2017 and January 2018 to March 2018. All procedures were approved by the Institutional Animal Ethics Committee (IAEC: GADVASU/2017/IAEC/40/19)

2.1. Chemicals and reagents

Analytical Reagent grade chemicals of Sisco Research Laboratories, Sigma and BR Biochem were used to conduct the experiments. Distilled water (DW) from Milliopore purification system (RO/Synergy) was used for the preparation of reagents.

2.2. Experimental design

Roosters (36–40 weeks old) were kept in individual cages and were given poultry feed and water ad libitum. Experiment was conducted on two generations. The RIR (n = 80) and Aseel (n = 50) roosters of first generation were evaluated for semen attributes and fertility. Based on semen and fertility analysis, selected roosters were divided into high and low fertile groups (Mavi et al., 2017, 2018). Polyclonal antibodies were raised in rabbits against sperm proteins (pooled from sperm extracts of ten roosters per breed) from the highly fertile first generation roosters. Chicks of next generation of selected roosters (Aseel, n = 22; RIR, n = 19) were also evaluated for semen characteristics/fertility and divided into high and low fertile groups (Table 1). Polyclonal antibodies raised against sperm proteins of high fertile first generation roosters were tested for characterization of fertility associated sperm proteins in the second generation.

2.3. Semen collection, evaluation and fertility trial

A single ejaculate of semen was collected from each of the roosters (first and second generation) twice a week using the abdominal

Table 1
Comparison between Aseel and RIR breeds on the basis of semen attributes (Mean ± SE).

S.no.	Semen Attributes	Aseel		RIR	
		G-I (> 50%)	G-II (< 50%)	G-I (> 50%)	G-II (< 50%)
1.	Motility	$73.6^a \pm 1.0$	$26.5^b \pm 2.2$	$85.3^a \pm 2.1$	$37.4^b \pm 1.7$
2.	Viability	$71.8^a \pm 1.0$	$29.7^b \pm 2.7$	$85.3^a \pm 0.8$	$37.3^b \pm 1.0$
3.	HOST	$64.6^a \pm 2.5$	$15.7^b \pm 1.9$	$85.0^a \pm 0.5$	$31.5^b \pm 1.6$
4.	Partially Damaged Acrosome	$65.3^a \pm 2.0$	$10.1^b \pm 1.3$	$32.3^a \pm 1.0$	$19.8^b \pm 1.1$
5.	Fully Damaged Acrosome	$22.3^a \pm 3.6$	$82.6^b \pm 0.6$	$11.6^a \pm 0.9$	$73.2^b \pm 1.4$

Superscripts indicate difference at 5% level with in the columns.

massage method (Burrows and Quinn, 1937). Semen was evaluated for different semen characteristics such as motility (subjective), viability (eosin-nigrosin staining), membrane integrity (50 mOsm/kg hypo-osmotic solution) and acrosome integrity (giemsa staining). Five hens per rooster were artificially inseminated. Eggs were collected and about 20 to 25 eggs/hen/chick/trial were incubated at 37 °C for 21 days. Eggs were candled on the 18th day of incubation to observe development of embryo and percent fertility was calculated as follow. Four hatches were set for each chick.

$$\text{Percent Fertile} = \frac{\text{Total number of fertile eggs}}{\text{Total number of eggs set}} \times 100$$

2.4. Producing hyper-immune serum

Semen of high fertile group was pooled, washed twice with PBS, pH 7.4 and proteins were extracted by suspending sperm pellet in 0.5 ml of 2% SDS solution in 62.5 mM Tris – HCl (pH 6.8). Sperm suspension was sonicated at 20 W for 3 x 20 s, centrifuged at 9615 *g* for 15 min. The pellet was discarded and sperm extract (SE) was concentrated through 3 kDa protein concentrators (Millipore) and stored in aliquots at −20 °C until further use. Rabbits were purchased from Animal house, Institute of Microbial Technology, CSIR, Chandigarh (approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India) and the experiment was performed in accordance with the guidelines approved by IAEC. Four adult male rabbits were divided into two groups (two rabbits per group i.e. G–I and G—II). The rabbits of the G–I and G–II groups were immunized with sperm extracts of high fertile Aseel and RIR roosters, respectively. Freund's complete adjuvant (FCA) was used for primary immunization and Freund's incomplete adjuvant (FIA) for subsequent boosters. About 200 μg protein/kg body weight emulsified with complete Fruend's adjuvant was injected intramuscularly on Day 0 (Day of first immunization treatment). Two boosters of the same dose mixed with incomplete Fruend's adjuvant were administered on Days 14 and 28, respectively. Blood was collected from the ear vein before each immunization and after 15 days of second booster. The blood was kept tilted for 2 h and centrifuged at 4 °C for 20 min at 865 *g* to collect serum.

2.5. Confirmation of presence of antibodies against sperm proteins in rabbit blood serum using an ELISA

A high binding 96 U bottom well ELISA plate (BR BIOCHEM, Life Sciences) was coated with 100 μl of poly-L-Lysine (MP biomedicals) by incubating at 37 °C for 1 h. The plate was washed twice with PBS containing 0.2% Tween 20 (PBS-T) and incubated with 100 μl of antigen (Sperm extracts of high fertile groups) overnight at 4 °C. The plate was washed thrice with PBS-T and incubated with 300 μl of 2% BSA solution for 2 h at 37 °C. The plate was washed thrice with PBST and coated with 100 μl of anti-serum serially diluted with PBS and incubated at 37 °C for 2.5 h. After washing, 100 μl of goat anti rabbit HRPO conjugate (1:10000, Genexbio) was added in all the wells and incubated for 3 h at 37 °C. The plate was again washed and 100 μl of OPD-H_2O_2 was poured and incubated for 20 min in a dark area of the laboratory. After 20 min, the reaction was stopped with 50 μl of 5N- H_2SO_4. Absorbance was quantified using an ELISA reader (Tecan) at 492 nm. The antibody titer was calculated from the maximum absorbance value at 492 nm. Percent positivity was also calculated using the formula:

$$\%\text{positivity} = \frac{\text{Mean absorbance of treated IgG - Mean absorbance of control IgG}}{\text{Mean absorbance of control IgG}} \times 100$$

2.6. Purification of IgG from serum

The IgG fraction containing anti- sperm protein antibodies (Anti-SP-IgG) of Aseel and RIR roosters was purified from rabbit blood serum using purification kit (Mol bio Himedia) as per manufacturer's instructions. The column was equilibrated with 5X resin bed volume of binding buffer. Then 5.0 ml of the serum was poured through the top of the column. Sample and resin were kept in contact for at least 15 min before removing the bottom cap and flow through was collected. The column was washed two or three times with 5.0 ml of binding buffer (Phosphate buffer saline: 0.1 M $NaHPO_4$, 0.15 M NaCl, pH 7.2–8.0) to eliminate all the proteins not retained in the column. Column was washed with binding buffer until the absorbance at 280 nm of the eluent reached the baseline. Bound IgG was eluted with 5.0 ml of elution buffer (0.1 M glycine, pH 2–3). Individual 1.0 ml fractions of elute were collected and neutralized by the addition of 0.15 ml of neutralization buffer (1 M Tris buffer, pH 8.5–9.0). Protein concentration was monitored by measuring the absorbance at 280 nm and combined the fractions with highest absorbance. The pooled IgG fraction was concentrated by passing through 3 kDa protein concentrators (Millipore) at 10,000 *g* for 30 min at 4 °C and quantity of protein was determined (Lowry et al., 1951). The purified IgG against Aseel and RIR sperm proteins was termed as anti-SPA-IgG and anti-SPR-IgG.

2.7. SDS PAGE and immuno-blotting of sperm proteins

Sperm protein extracts of Aseel (high fertile, $n = 10$; low fertile, $n = 12$) and RIR (high $n = 12$; low, $n = 7$) roosters of the second generation were prepared as described in Section 2.4. About 100 μg of protein was loaded to each well and a dis-continuous SDS-PAGE was performed using 12% acrylamide gels (Laemmli, 1970). The gel was stained with 0.5% CBB for 2–4 hrs and de-stained with de-staining solution until the blue bands appeared against a clear background. The image analysis and molecular weight determination was conducted using the Gene Snap Image Acquisition software (Syngene).

Sperm proteins of each rooster of both breeds resolved on 12% SDS-PAGE were transferred electrophoretically (Towbin et al., 1979) to nitrocellulose membrane (NCM, HiMedia) using the Pierce G2 fast blotter (Thermo Scientific). After electrophoresis, the gel was removed from the plates and kept in transfer buffer along with the NCM for 30 min to remove excess SDS from the gel. Stack was prepared in the following sequence: two absorption pads, gel, NCM, two absorption pads. The complete stack was kept on the G2 fast blotter between cathode and anode. Blotting was conducted for 15 min at 25 V and 1.3 A. After transfer, membrane was stained with ponceau dye (1–2 min) to assess for the efficiency of transfer. The membrane, after transfer was incubated in 2% BSA for 2 h at room temp on platform rocker for blocking the nonspecific binding sites. The membrane was washed thrice with PBS-T (each washing for 15 min) and incubated with 1: 5000 diluted purified anti-SPA-IgG or anti-SPR-IgG at 4 °C overnight. The membrane was washed thrice with PBS-T and incubated with 1:20,000 diluted goat anti-rabbit HRPO conjugate (Bangalore Genei) for 45 min at 37 °C. After washing, the protein antibody reaction was detected by incubating the membrane with substrate containing 0.06% DAB and 0.06% H_2O_2 for 5 min. The reaction was terminated by washing the membrane with DW. The image analysis and molecular weight determination was conducted using Gene Snap Image Acquisition software (Syngene).

2.8. Mass spectrometry

Bands of 30/20 kDa (Aseel) and 25/20 kDa (RIR) were cut from acrylamide gel and submitted to Central Instrumentation Facilities (CIF), University of Delhi South Campus, Benito Juarez Road, Dhaula Kuan, New Delhi for mass spectrometry analysis using MALDI-TOF. To extract protein from the gel, destained gel slices were stored overnight in 10% acetic acid and centrifuged at 9615 *g* for 5 min to remove supernatant. Samples were washed thoroughly with distilled water five times, vortexed for 3 min and added 50 μl of 1 M ammonium bicarbonate solution (at 4 °C), 475 μl of distilled water and 500 μl of ACN. Vortex the mixture for 5 to 10 min until the color was removed. Again 500 μl of CAN was added twice to the gel slices and kept for 5 min to remove water, and was centrifuged for 3 s at 9615 *g* to remove ACN properly. Samples were air dried for half an hour at room temperature in eppendrof tubes and rehydrated with 10 to 20 μl of trypsin solution prepared in 25 mM ammonium bicarbonate and incubated on ice for 20 to 30 min until trypsin was absorbed. After gel slices were completely rehydrated, a minimal amount of 25 mM ammonium bicarbonate was added to cover gel slice in tube and incubated at 37 °C in water bath for overnight, centrifuged for 3 min at 15,000 *g*. The remaining gel slices were vortexed with 50 μl of extraction solution for 5 min. Extracted supernatant was collected in fresh eppendrof tube. There was 50 μl of 40% ACN solution added to gel slices for peptide extraction and then samples were vortexed for 5 min. Centrifuged the gel containing ACN at 11,634 *g* for 30 s. There was collection of the supernatant and mixing with the first extracted supernatant.

Search parameters for MALDI-TOF MS analysis of peptides were MS/MS ion search, trypsin digestion, monoisotopic mass value, unrestricted protein mass ± 1.2 Da peptide mass tolerance and number of queries varied from 164 to 261. Identified peptide sequences were aligned and compared with the sequences of different proteins using BLAST search engine.

2.9. Statistical analysis

The mean and standard error were calculated using Microsoft excel program. Significant differences (5% level) between the two groups for each breed of various semen attributes were tested by *t*-test using SPSS16 program (Student version for windows, SPSS Inc.233 South Wacker Drive, 11th floor Chicago, IL 60606-6412). Normality of the data was assessed using the Shapiro-Wilk test and homogeneity of variances was evaluated using the Levene test.

3. Results

3.1. Confirmation of anti-sperm protein antibodies in rabbit blood serum

Immunization of rabbits with sperm proteins of Aseel and RIR breeds resulted in 87.7% and 110.5% positivity of raised antibodies after 3rd immunization, respectively.

3.2. Characterization of sperm proteins by SDS- PAGE

A total of 16 and 10 bands ranging from 18 to 130 kDa and 20 to 110 kDa, were separated using the 12% acrylamide gels in Aseel and RIR roosters, respectively (Figs. 1–4, Table 2). All the bands, however, were not assessed for all Aseel and RIR roosters. Comparison between Aseel and RIR roosters indicated that proteins of 130, 70, 44, 38, 32, 27 and 18 kDa were specific to Aseel roosters. Three proteins of 110, 75, 70 kDa and two proteins of 38, 32 kDa were specific to high and low fertile roosters, respectively. Two proteins of 62 and 18 kDa were detected in greater numbers (100%) of low fertile compared to high fertile Aseel roosters (30% and 20%). On the contrary, proteins of 50 and 30 kDa were detected in a greater number of high fertile (100%) compared to low fertile Aseel roosters (83.3%, 41.7%). Comparison of proteins based on fertility indicated the presence of 55 and 50 kDa proteins only in high and low fertile RIR roosters, respectively.

3.3. Characterization of proteins related to fertility of roosters using immunoblotting

Among 16 (Aseel) and 10(RIR) bands separated using SDS-PAGE, eight and ten bands reacted with anti-SPA-IgG and anti-SPR-IgG, respectively (Figs. 5–8 and Table 3). There were differences in number of bands reacting with anti-IgG among the roosters of

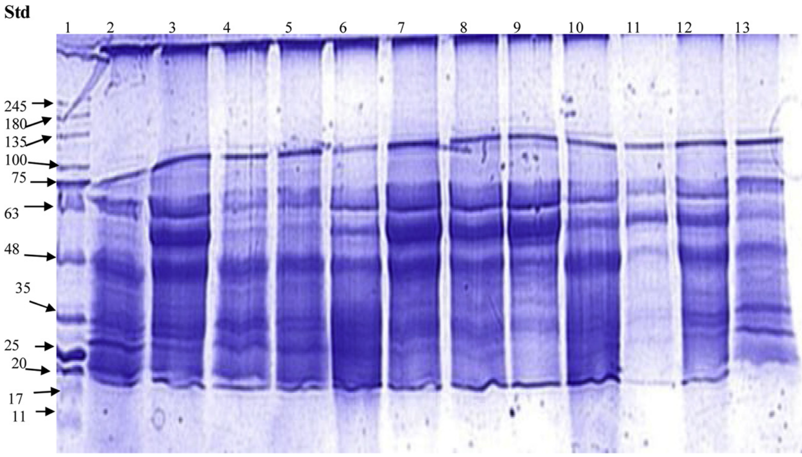

Fig. 1. SDS-PAGE of sperm extracts of roosters of the Aseel breed with < 50% fertility rate; Sperm extracts were separated on 12% acrylamide gels and stained with commassie brilliant blue; 1: standard, 2-1 3: number of chicks (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

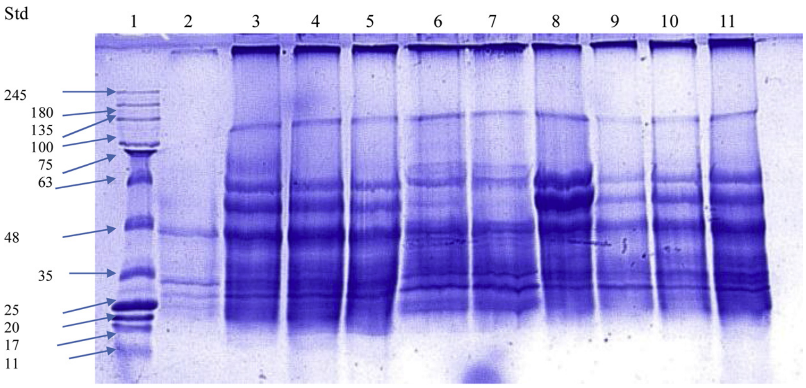

Fig. 2. SDS-PAGE of sperm extracts of roosters of the Aseel breed with > 50% fertility rate; Sperm extracts were separated on 12% acrylamide gels and stained with commassie brilliant blue;1, standard, 2–11, number of chicks (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

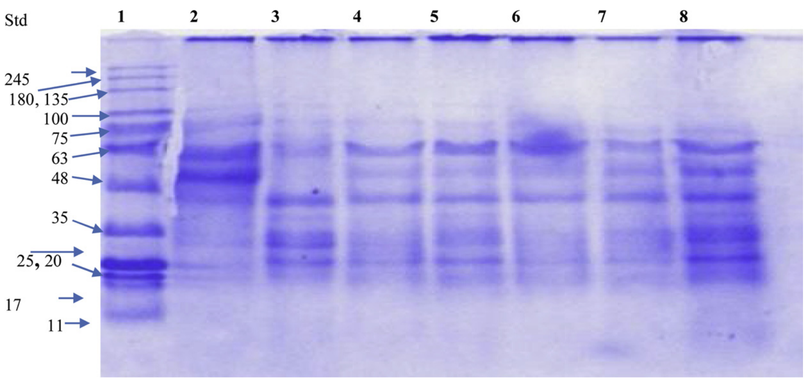

Fig. 3. SDS-PAGE of sperm extracts of roosters of the RIR breed with < 50% fertility rate. Sperm extracts were separated on 12% acrylamide gels and stained with commassie brilliant blue.1: standard, 2-8: number of chicks (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

both breeds. A 30 kDa (Aseel) and 55/20/18 kDa (RIR) proteins reacted with the respective anti –IgG only in high fertile roosters. There were 50, 40 kDa (Aseel) and a 50, 35 kDa (RIR) proteins, however, that reacted to respective anti-IgG only in low fertile roosters. A 20 kDa and 30/25 kDa proteins were recognized with respective anti-IgG in a greater number of high fertile Aseel (50% compared 44%) and RIR roosters (100% compared with 57.14%, 14%) compared to low fertile roosters. Two proteins of 55/35 kDa

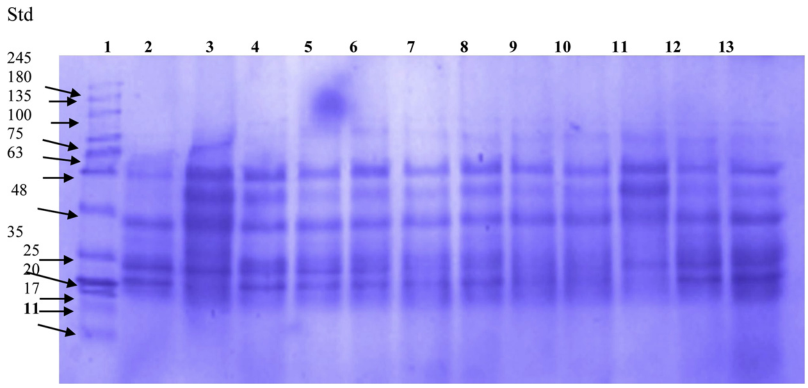

Fig. 4. SDS-PAGE of sperm extracts of roosters of the RIR breed with > 50% fertility rate; Sperm extracts were separated on 12% acrylamide gels and stained with commassie brilliant blue;1: standard, 2-13: number of chicks (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Distribution (%) of different sperm proteins separated using SDS-PAGE based on breed and fertility of roosters.

S. No.	Molecular Weight of Protein (kDa)	Aseel		RIR	
		> 50% Fertility Rate	< 50% Fertility Rate	> 50% Fertility Rate	< 50% Fertility Rate
1	130	90	100	0	0
2	110	0	100	100	100
3	75	0	100	100	100
4	70	0	100	0	0
5	62	30	100	100	100
6	55	100	100	100	0
7	50	100	83.3	0	100
8	46	100	100	100	100
9	44	100	83.3	0	0
10	40	100	83.3	100	100
11	38	100	0	0	0
12	35	100	100	100	100
13	32	100	0	0	0
14	30	100	41.7	100	100
15	27	100	100	0	0
16	25	100	100	100	100
17	20	100	100	100	100
18	18	20	100	0	0

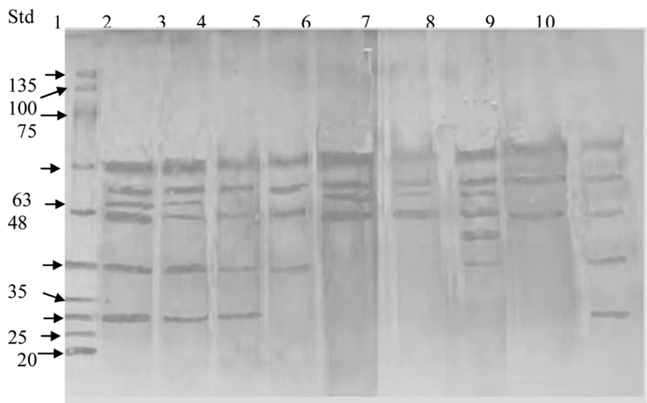

Fig. 5. Immunoblotting of sperm extracts from roosters of that Aseel breed with < 50% fertility rate with rabbit antibodies raised against sperm extracts of > 50% fertility rate chicks; Sperm extracts were separated on 12% acrylamide, transferred to a nitrocellulose membrane and reacted with sperm extract antibodies 1: standard; 2-10: number of chicks.

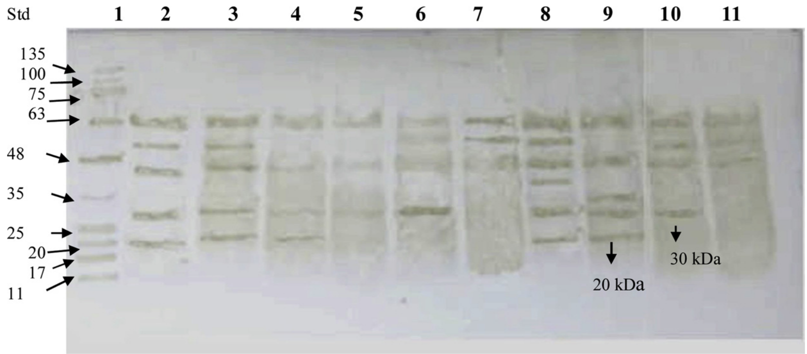

Fig. 6. Immunoblotting of sperm extracts from roosters of the Aseel breed with > 50% fertility rate with rabbit antibodies raised against sperm extracts of high fertile chick. Sperm extracts were separated on 12% acrylamide, transferred to a nitrocellulose membrane and reacted with sperm extract antibodies; 1: standard; 2-11: number of chicks.

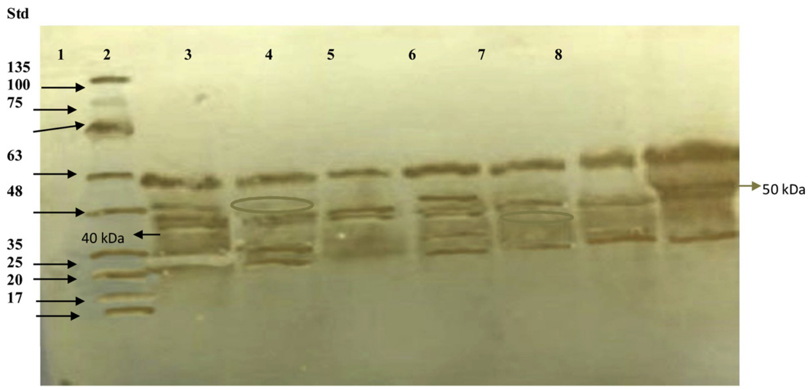

Fig. 7. Immunoblotting of sperm extracts from rooster of the RIR breed with < 50% fertility rate with rabbit antibodies raised against sperm extracts of > 50% fertility rate chicks; Sperm extracts separated on 12% acrylamide, transferred to a nitrocellulose membrane and reacted with sperm extract antibodies; 1: standard; 2-8: number of chicks.

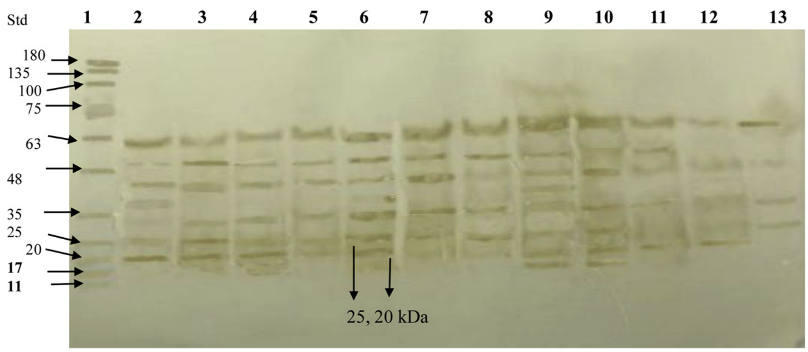

Fig. 8. Immunoblotting of sperm extracts from roosters of the RIR breed with > 50% fertility rate with rabbit antibodies raised against sperm extracts of high fertile chick; Sperm extracts were separated on 12% acrylamide, transferred to a nitrocellulose membrane and reacted with sperm extract antibodies; 1: standard; 2-13: number of chicks.

(Aseel) and a 40 kDa (RIR) protein were detectable with respective anti-IgG in greater numbers of low fertile Aseel (100%, 66.66% compared with 70%, 10%) and RIR (86% compared with 25%) roosters compared to the counterparts in this study.

Mass spectrometry (MS) analysis indicated there were matching of 20, 30, and 20, 25 kDa proteins of Aseel and RIR roosters with there being an immunoglobulin kappa chain variable, phospholipase A_2 (PLA2), hypothetical N332-08551 partial and cystatin like partial proteins with a top score of 41, 46, 52 and 43, respectively (Table 4). A total of 6.5, 6.0, 13 and 6.5 peptides of 20, 30, 20 and 25 kDa bands were matched to previously identified proteins from the database, which had coverage of 57%, 44%, 87% and 53% of whole protein sequence, respectively. During this study, antigens of 30/20 kDa and 20/25 kDa were observed to be associated with a greater fertility rate of Aseel and RIR roosters.

Table 3
Distribution (%) of different sperm antigenic proteins detected using immunoblotting with antisperm protein IgG based on breed and fertility of roosters.

S. No.	Molecular Weight of Protein (kDa)	Aseel		RIR	
		> 50% Fertility Rate	< 50% Fertility Rate	> 50% Fertility Rate	< 50% Fertility Rate
	62	100	100	100	100
	55	70	100	83.3	0
	50	0	55.6	0	57.1
	46	100	100	100	100
	40	0	11.1	25	85.7
	35	10	66.6	0	28.4
	30	80	0	100	57.1
	25	0	0	100	14.3
	20	50	44.4	91.7	0
	18	0	0	0	0

4. Discussion

Protein bands, separated by SDS-PAGE ranged from 18 to 130 kDa and 20 to 110 kDa in Aseel and RIR breeds, respectively. Shanmugam et al. (2016) could resolve only low molecular weight proteins (< 95 kDa) in sperm extracts of a PD-3 line and white leghorn control line of chickens. There was variation in sperm proteins among Aseel and RIR breeds in the presentis study. Inconsistent with data from previous studies (Shanmugam et al., 2016), in the present study there was not any differences in proteins among the two layer lines. Difference in findings in the present and previous studies may be due to breed difference. The SDS-PAGE and Immunoblotting of sperm proteins of Aseel and RIR roosters confirmed the association of sperm proteins with fertility and subfertility of roosters. Qualitative analysis in a previous study allowed for identification of 1165 proteins that were primarily associated with oxido-reduction mechanisms, energy processes, proteolysis and protein localization of chicken semen (Labas et al., 2014). There was a comparative analysis conducted between the most and the least fertile males. The enzymes involved in energy metabolism, respiratory chain or oxido-reduction activity were over-represented in spermatozoa of the most fertile males. The seminal plasma of the high and low fertile males differed also in many proteins (e.g., ACE, AvBD10 and AvBD9, NEL precursor, acrosin; Labas et al., 2014).

The MS analysis indicated there was a matching of 30 kDa sperm protein of Aseel roosters with phospholipase A2 in the present study. In mammals, sperm acquire progressive motility and prime the signaling pathways that eventually regulate capacitation during epididymal transit. Spermatozoa attain full fertilization potential in vivo only after capacitation: a final maturation process occurring in the female reproductive tract (Stival et al., 2016). Thereafter sperm binds to the zona pellucida, followed by the physiological acrosome reaction (AR), which is essential for sperm-oocyte fusion. Only fully capacitated spermatozoa can undergo AR and bind to the zona-intact oocyte (Jungnickel et al., 2003). The acrosome reaction is also very important for successful fertilization in chickens (Okamura and Nishiyama, 1978; Lemoine et al., 2011). The acrosome reaction involves fusion of plasma membrane with the outer acrosomal membrane and results in the release of proteolytic enzymes that hydrolyze the inner perivitelline layer surrounding the oocyte (Bakst and Howarth, 1997; Okamura and Nishiyama, 1978). Avian spermatozoa do not undergo capacitation or the motility hyper-activation process prior to fertilization (Lemoine et al., 2008), although spermatozoa are located for a very long time in the female oviduct before oocyte penetration. The PLA2s are likely to be important, because of the abundant amounts of these proteins in the male reproductive organs (Koizumi et al., 2003; Bao et al., 2004; Masuda et al., 2005; Roldan and Shi, 2007). Results of different studies with mammals indicate one or several uncharacterized PLA2s have an important role in capacitation, AR, and the early stages of fertilization, including sperm binding and sperm-oocyte fusion (Roldan and Fragio, 1993; Pietrobon et al., 2005; Roldan and Shi, 2007; Stival et al., 2016). Results of several biochemical studies indicate the presence of one or several low-molecular weight Ca2 + -dependent sPLA2-like proteins in spermatozoa of different species (Lessig et al., 2008). The presence of mouse group X sPLA2 (mGX), which is released in an active form during capacitation through spontaneous AR has been reported (Escoffier et al., 2010). Matching of 30 kDa protein of Aseel sperm to PLA_2 in mass spectrometry analysis, therefore, indicates there is the presence of this enzyme of physiological significance in chickens.

The results of the MS analysis indicate there was a 20 kDa sperm protein in Aseel as the immunoglobulin kappa chain variable. These proteins are large abundances in spermatozoa and oocytes with unknown functions (Joho et al., 1990). Organization of the K light chain genes has been previously studied in germ line (sperm) and somatic (embryo) tissues (Joho et al., 1990). Results, however, did not indicate there was rearrangement of variable region genes (or "minigenes") during early embryogenesis. Detection of 20 kDa Aseel sperm protein, however, as an immunoglobulin kappa chain variable indicates there is a need to identify its function in chicken sperm.

Results from the MS analysis in the present study indicated that there was a 25 kDa protein of RIR spermatozoa that was a cystatin like partial protein. The cystatins are a superfamily of cysteine proteinase inhibitors that consist of three evolutionarily related families (Barrett, 1986). It was established in vitro that cystatins are specific inhibitors of papain-like cysteine proteinases such as the mammalian cathepsins B, H, L, and S. Some researchers have suggested that cystatins may have an important regulatory role in normal body processes (Buttle et al., 1990; Lah et al., 1993; Calkins and Sloane, 1995; Keyszer et al., 1998). Ying et al. (2002) used

Table 4
Mass Spectrometry analysis of Sperm proteins of Aseel and RIR roosters.

S. No.	Molwt (kDa)	Sequence detected	Sequence matched to	Score	Protein sequence coverage (%)
1	30A	MNAFQTVFLLAVGLSVAHSLDYKALTQFRNMIICLMPDSWPALDYADYGC51YCGWGGSGTPVDDLDR	phospholipase A2 [Fundulusheteroclitus	46	44
2	20A	ASQSIDKYLNWYQQKPGKFSGGGSGTDFTLTISSLQPEDFATYFCQHSYSIPLTFGGGTK	Immunoglobulin kappa chain variable region, partial [Homo sapiens]	41	57
3	20R	VRVSVSTCVCVSVCPSVCPCVHLSVRPSLC VCLSVCPCVHPSVRASICPCVHLSVCLSVCVSVCPSVCPSVCPCVSPSLCPSVCPCVRMSVHLSVYLCLS HLSVHVSVCPCVRPSVCPSLCPCVRVCVR	Hypothetical protein N332_08551, partial [Mesitornis unicolor]	52	87
4	25R	RQVVAGLKYIIEAEIARTTCPKPAADLQSCAFNDAPQMAEHTTCNFVVYTVPWLNKMQLLDSSCH	cystatin-like, partial [Buceros rhinoceros silvestris]	43	53

differential display-reverse transcriptase-polymerase chain reaction to examine Sertoli cell gene expression in mice and cystatin SC (cystatin-related gene expressed in sertoli cells) as well as cystatin TE-1 (cystatin-related gene highly expressed in testis and epididymis) were isolated. The findings in this previous study further suggested that cystatins SC and TE-1 have a very specialized role in the testis and epididymis (Cornwall et al., 2011; Parent et al., 2011). Feyisa et al. (2018) reported that cervical CST3 may prevent precocious capacitation and acrosome reaction, thus preserving sperm fertilizing capacity before it reaches the fallopian tube. Additionally, CST3 may function to facilitate sperm entry into the upper reproductive tract by enhancing sperm motility. Hence, the role of cystatin like partial protein in chicken sperm function can also be predicted, evaluated and confirmed in future.

The sequence of a protein with a molecular weight of 20 kDa of RIR sperm matched to the hypothetical N332-08551 partial protein using the blast procedure. This protein was sequenced by Zhang and Li (2014, unpublished data) in the Mesitornis unicolor bird while studying genomic evolution of the birds. This hypothetical protein is a protein where the existence has been predicted but there is lack of experimental evidence for its presence *in vivo*. Matching of 20 kDa Aseel sperm protein to the hypothetical N332-08551 partial protein of Aves indicates a need for further evaluation of the gene expression and role of this protein in chicken reproduction/fertility.

5. Conclusions

There is difference between two groups in semen attributes and fertility of the Aseel and RIR breeds. Results of the present study suggest that proteins identified and characterized based on fertility rate of roosters may be developed as potential markers for fertility evaluation of chickens.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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