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Research article

ARTIFICIALLY GENERATED MALE-SPECIFIC RABBIT ANTIBODY AGAINST DBY-MULTI-EPITOPE FUSION PROTEIN AND ITS IMMUNOREACTIVE EXAMINATION

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DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked (*DBY* or *Ddx3y*) is a candidate gene for male-specific antigen. The *DBY* gene detected in capacitated mouse sperm codes putative ATP-dependent RNA helicase. The objective was to produce anti-predicted DBY multi-epitope fusion protein antibody, which could be used to determine male specificity of DBY. Epitope prediction is to aid the design of molecules that can mimic structure and function of a genuine epitope, is a useful tool in protein molecule design. This study predicted the DBY epitopes, prepared rabbit poloclonal antibody against DBY multi-epitope fusion protein, then investigated its immunoreactivity. The fusion protein used as the antigen consisted of three regions of DBY with greatest divergence from other family members, cloned together in-frame (with a His tag to facilitate purification). The resulting antibody recognized both the DBY1-2-3 fusion protein and an endogenous DBY protein of the same size. Furthermore, DBY protein was present (Western blot) in testis, male mouse splenocytes and brain, whereas a weaker band was present in the female brain and splenocytes, and finally, ovary produced only a barely visible protein band. Optical density of DBY protein was higher for males versus corresponding tissues from females. Finally, positive signals of DBY1-2-3 antibody were present on only ~60% of mature murine sperm (based on immunofluorescent staining and flow cytometry), in accordance with the expected proportion of Y-bearing sperm. We hypothesized that our antibodies recognized a specific epitope present in subpopulations of mouse sperm. Therefore, we concluded that anti-DBY1-2-3 antibody could be an alternative way of producing antibodies to DBY protein. Furthermore, this novel DBY antibody against a multi-epitope artificial antigen has potential for both investigating male-specific binding of DBY and as a new method of sex selection.

Key words: DBY, antibody, spermatozoa, gender selection

INTRODUCTION

The SDM antigen (serologically detected male antigen), part of the H-Y antigen family, is a group of surface proteins on normal male cells but not female cells [1-4].

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The SDM antigen has a potential for sexing embryos and discriminating between X- and Y-bearing sperm [2,5-7], due to male specificity of SDM antigen. However, there is a paucity of recent studies regarding the SDM antigen and its potential for use in sex selection.

Male enhanced antigens (Mea), sex determining region Y (*Sry*) [8], *Smcy* [9] and DEAD (Asp-Glu-Ala- Asp) boxpolypeptide3,Y-linked (*Ddx3y*, or *Dby*) [10] are candidate genes for male-specific antigens of mammals. In birds, the heterogametic female sex is serological sex-specific (Sxs) antigen ("H-Y antigen") positive [11], chromo helicase DNA gene within W sex chromosomes (CHD-W) is unique to female birds, making sex identification possible [12]. The *DBY* gene codes for putative ATP-dependent RNA helicase, and is the only gene detected in capacitated mouse sperm. It was reported that *DBY* may have both housekeeping and testis-specific functions [13], , and that translation of *DBY* occurred only in male germ lines [14]. It has homologous genes, namely *Ddx3x* on the X chromosome and *D1Pas1* on mouse chromosome 1 [15]. , with 90 and 87% identity (amino acid level), respectively, with these two genes. Due to substantial homology among DBY, DDX3X and D1Pas1, a DBY-specific antibody is difficult to obtain. Recently, polyclonal antibodies against epitopes of Mea and DBY were developed in rabbits; the resulting sera separated nearly 95% of Y-bearing sperm [16], and limited viability and motility of Y-bearing pig sperm. Consequently, this approach has the potential for investigating SDM as a male-specific antigen, with potential applications in sperm or embryos (i.e. assisted reproductive technologies).

This study describes the use of a novel fusion protein to produce antibodies to mouse DBY/DDX3Y protein. In that regard, we developed an anti-DBY multi-epitope fusion protein antibody to investigate male specificity of DBY, with potential to develop a new method for sex selection.

MATERIAL AND METHODS

Epitopes screening of DBY protein

The FASTA format of DBY protein sequence (accession ID CAA07483.1, *Mus musculus*) was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/protein/). Its secondary structure was predicted by DNAStar Protean software with the Gamier-Robson program (DNASTAR, Inc, Madison, WI, USA)[17]. Its B-cell epitope was analyzed using online ABCpred servers (http://www.imtech.res.in/raghava/abcpred/) [18] and BepiPred 1.0 servers (http://www.cbs.dtu.dk/ services/BepiPred) [19]. Sequence alignment of DBY and DDX3X were done with the ClustalW program (by selecting DBY-specific epitopes). Screening for DBY epitopes was based on DBY and DDX3X antigen's primary amino acid sequence, Pymol 3D structure, and or other protein characteristics (hydrophilicity, accessibility and flexibility).

Prokaryotic expression and purification of DBY multi-epitope fusion protein

Total RNA was extracted from brain tissue of 6-week-old male Kunming mice (an outbred mouse stock, with a high ratio of gene heterozygosis, derived from Swiss albino mice) using a TRIzol kit (Invitrogen Corp, Carlsbad, CA, USA), and reverse-transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI, Fermentas, Vilnius, Lithuania). Three gene-encoding antigenic peptides containing corresponding epitopes [22-138 aa (DBY1); 461-492 aa (DBY2); and 585-631 aa (DBY3)] were amplified by PCR, corresponding to their primer pairs (Table 1). This was done using conventional PCR methods (35 cycles with 94°C for 30 s, 55°C for 1 min, and 72°C for 35 s). Amplified products were gel-purified and sequenced.

Table 1. Primer pairs of DBY antigenic peptides

	Primer sequence	DBY antigenic peptides	DBY epitopes	
DBY1-F	5'-GCGGAATTCGACCTGAAATCATCAGAT-3'	22-KSSDNQNGGGN…DRSDEDDWSKP-138 357 bp	36—KGRYIPPHLRNRETSK—51	EcoR I
DBY1-R	5'-CCGGAGCTCTGGTTTTGACCAGTCATC-3'			Sac I
DBY2-F	5'-GGCGAGCTCTTTCAAGAAAGATATGCG-3'	461-FQERYACTSIHGD…ALHQFRSGRKPILV-492 105 bp	486-FRSGRKPILVATAVA-500	Sac I
DBY2-R	5'-GGGTCGACCACTAAAATTGGCTTTCG-3'			Sal I
DBY3-F	5'-CGGTCGACGGACGTTCTAAAAGCAGA-3'	585-GRSKSRFSGGFG…SRSSGSSHNRGFG-631 141 bp	607-ANAGFNSNRANSSR—620	Sal I
DBY3-R	5'-GCGAAGCTTACCAAATCCTCTGTTGTG-3'			Hind III
β-Actin F	5'-CCTAAGGCCAACCGTGAAAAGATG-3'	430 bp	430bp	
β-Actin R	5'-ACCGCTCGTTGCCAATAGTGATG-3'			

Rstriction enzyme-digested products of *DBY1*, *DBY 2*, and *DBY 3* (equal ratios, Table 1) were ligated with T4 ligase (TOYOBO, Osaka, Japan).The ligated (recombinant) DNA was subsequently amplified using PCR with F1 and R3 primers (Table 1), as described above. The PCR products were purified with agarose gel electrophoresis, digested with EcoR I and Hind III, and sub-cloned into an pET-32a expression vector (Novagen, Hercules, CA, USA).The recombinant plasmid pET-32a-DBY1-2-3 was expressed as a His-tagged fusion protein in *Escherichia coli* BL21, according to the pET System Manual. The fusion protein was purified with a Ni-NTA-Sefinose Column (Bio

BasciInc, Toronto, ON, Canada) and its concentration determined with a Coomassie Brilliant Blue G Microassay (Tiangen, Beijing, China).

Preparation and Purification of Polyclonal antibody against fusion protein with DBY epitopes

New Zealand white rabbits (2.5-3.0 kg) were immunized by im injections of fusion protein complex (250 ug/kg), four times (2-week intervals), with an equal volume of Freund's adjuvant (Sigma-Aldrich Co., St. Louis, MO, USA, complete and incomplete for first and subsequent immunizations, respectively). Rabbits in the control group were injected with an equivalent volume of PBS (pH=7.0). Sera were purified using a Protein A Sepharose 4 FF Antibody Purification Kit (Bio-Zhongkechenyu, Beijing, China), according to the manufacturer's instructions.

Male specific binding activity of DBY1-DBY2-DBY3 antibody

Splenocytes and tissue samples (brain, testis and ovary) were collected from male and female Kunming mice, and lysed with ice-tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration in supernatant of the lysates was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The DBY1-2-3 antibody (~9.5 mg/mL) was tested using a western blot analysis, as described [4] using approximately 10.0 mg/mL proteins from the above cells and tissues as antigens, and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Pierce Biotechnology, Rockford, IL, USA) as secondary antibody for signal detection.

Sperm from Kunning mice were incubated for 1 h at 37 °C with ~9.5 mg/mL DBY1-2-3 antibody, then centrifuged (1000 g for 10 min), washed with PBS, incubated with 1:5000 diluted FITC-conjugated goat anti-rabbit IgG for 1 h at 37 °C, and then washed with PBS. Normal serum was used as a negative control, whereas secondary antibodies only (FITC-conjugated goat anti-rabit IgG) as a background control in the detection system. Immunofluorescence and flow cytometry (FCM) of sperm cells was done as described [16] Quantitative analysis of immunofluorescence was performed by counting the number of positive and total sperm in five randomly selected fields of every section using Image Pro-plus software

RESULTS

Screening and expression of DBY antigenic peptide

Differences among sequences of the three proteins (DBY, DDX3X, and D1Pas1) were predominantly located at the N- and C-termini. Three DBY epitopes (Table 1) that matched DBY specificity criteria and had the most hydrophilic portions of the protein, were chosen based on comprehensive analysis of several potential epitopes,

structural characteristics, and sequence alignment of DBY and DDX3X, D1Pas1 protein sequences. These epitopes were extended to three antigenic peptide sequences (Table I), including differences among the three proteins.

The PCR products of DBY1-2-3 were 357, 105, and 141 bp, respectively (Figure 1A). The DBY1-2-3 ligated product was constructed using restriction enzyme digestion, ligated, and then amplified using PCR with F1 and R3 primers (Table 1). The DBY1-2-3 PCR product was purified with agarose gel electrophoresis, digested with EcoR I and Hind III, and sub-cloned into an expression vector (pET-32a). The recombinant DBY1-2-3-pET-32a was digested with EcoR I and Hind II; the resulting gel had two bands of plasmid and insert (Figure 1A), with the sequence of the latter consistent with DBY1-2-3.

Based on SDS-PAGE, optimized expression of recombinant plasmid DBY1-2-3-pET-32a was induced by IPTG, for 6 h at 30°C (Figure 1B). Soluble protein was purified using a Ni column purification kit (Figure 1C).

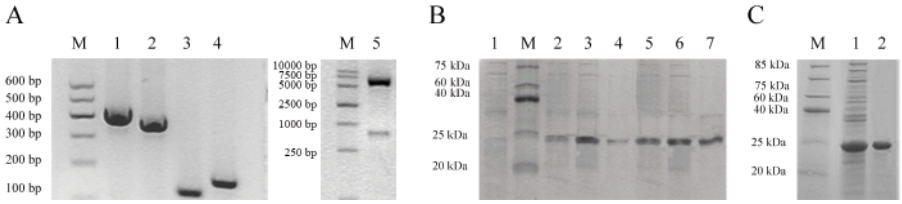

Figure 1. Expression of DBY1-DBY2-DBY3 fusion protein.
Agarose gel electrophoresis of PCR products of *DBY* DNA fragments encoding antigenic peptides containing corresponding epitopes **(A)**. 430, 357, 105, and 141 bp fragments were amplified for actin (1), DBY1 (2), DBY2 (3), and DBY3 (4), respectively. Recombinant Plasmid pET-32a-DBY1-2-3 DNA samples were double-digested with restriction endonucleoases EcoR I and Hind III. Positive clones (5) contained an insert fragment (603 bp) and pET-32a (5900 bp). The 600bP Plus DNA Ladder was used as a molecular weight maker (M). Lanes 1 to 7 represent SDS-PAGE analysis of expression of DBY1-2-3 **(B)**. DBY1-2-3 samples were induced by IPTG at 37°C for 0 h (1), 37°C for 1 h (2), 37°C for 3 h(3), 37°C for 6 h (4, 5), and 30°C for 6 h (6, 7); M, molecular weight size marker. The amount of expressed proteins differed according to conditions of IPTG induction. SDS-PAGE analysis was used to verify presence of purified protein **(C)**, DBY1-2-3 protein before purification (1) and DBY1-2-3 protein after purification (2).

Preparation and functional analysis of antibody against DBY1-2-3 antigenic peptide

Based on Western blot, the prepared rabbit DBY antibody specifically bound to soluble DBY1-2-3, which had a band of the expected size (25 kDa; Figure 2A). Furthermore, DBY protein of the expected size was detected (Western blot) in testis and in male mouse splenocytes and brain (Figure 2B), whereas there was a weaker band for female mouse brain and female mouse splenocytes, with a barely visible protein band detected for ovaries. There were apparent differences between males and females for corresponding tissues. Moreover, when comparing male and female

spleen/brain tissues there was a stronger signal in male tissues, indicating that the antibody preferentially detected DBY compared to DDX3X

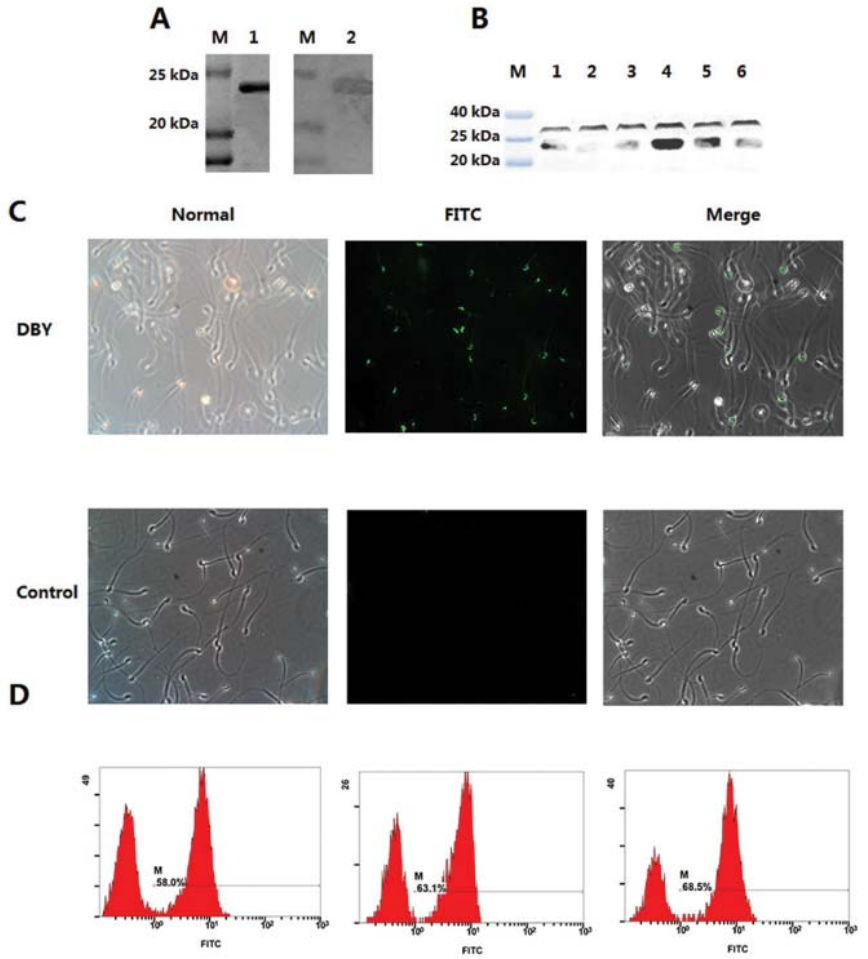

Figure 2. Functional analysis of antibody against DBY1-DBY2-DBY3 antigenic peptide. Western blot analysis of soluble DBY1-2-3 **(A)**. Lane 1 represents SDS-PAGE analysis of DBY1-2-3; Lanes 2 represents immunoblot analysis of DBY1-2-3 with its rabbit DBY1-DBY2-DBY3 antibody; and M was the molecular weight size marker. Specific binding activity of the DBY1-DBY2-DBY3antibody to DBY1-2-3 was detected with Western blot analysis **(B)**. Total proteins from cells and tissues were separated by SDS-PAGE and subjected to Western blot analysis using DBY1-DBY2-DBY3 antibody. Lane 1, testis; Lane 2, ovary; Lane 3, female splenocytes; Lane 4, male splenocytes; Lane 5, male brain; Lane 6, female brain. The upper band with equal intensity (in all lanes) was loading control (β-Actin). Immunostaining analysis of DBY on mouse sperm cell (C). DBY was detected in the head surface of sperm cell in the DBY1-DBY2-DBY3 antibody (green signals). However, signals were not detected in the negative control (normal rabbit sera). Anti-DBY1-2-3 antibody activity on mouse sperm measured by flow cytometry with three replicate analyses **(D)**. Mouse sperm were treated with rabbit DBY1-2-3 antibody. Secondary FITC-conjugated goat anti-rabbit IgG was added after sperm were incubated and washed.

The DBY1-2-3 antibody bound to the sperm head membrane (Figure 2C), whereas immunostaining was rarely present on the sperm tail. Furthermore, there were no apparent binding signals in mouse sperm when only normal serum and secondary antibodies were used. Interestingly, there were positive signals of DBY1-2-3 antibodies on approximately (58 to 61%) of mouse sperm (three repeated experiments), approximately equal to the expected ratio of 1:1 of X- and Y-bearing sperm [20]. Furthermore, based on flow cytometry, DBY1-2-3 antibody bound to approximately (58 to 68%) of mouse sperm (Figure 2D).

DISCUSSION

Candidate gene work for SDM antigen has been studied using various approaches, including identification of male-specific genes or proteins, in addition to preparation and testing of SDM specific antibodies using whole cells, cell lysis, or candidate proteins [21,22]. To date, nearly all approaches have been unsuccessful, although the immunological approach has had some promise. Perhaps there are differences in protein composition between X- and Y-bearing sperm for a minor component of the membrane with low antigenicity [21,23]. In the present study, the search for SDM antigen was based on comparison of protein sequences DBY and DDX3X, D1Pas1, screening and structure analysis of DBY epitope, which may produce an antibody with higher affinity and/or specificity. The fusion protein used as antigen consisted of three regions of DBY with greatest divergence from the rest of the family. The antibody produced recognized both the DBY1-2-3 fusion protein and an endogenous DBY protein of the same size. Therefore, we concluded that anti-DBY1-2-3 antibody could be an alternative way of producing antibodies against DBY protein.

There is a high degree of sequence similarity among *DBY/DDX3Y*, its X homolog *DBX/DDX3X*, and an autosomal homolog *D1PAS1*. Furthermore, *DBY/Ddx3y* is expressed across a broad range of tissues, whereas *DBY/Ddx3y* mRNA in mouse is expressed more much strongly in the brain and spleen than in the testis, where it is barely detectable (http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GD

S3142:1426439_athttp://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS3142:1452077_at;
http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS3142:1426438_at). The *Ddx3y* gene expression profiles of GEO Profiles database in NCBI was consistent with our Western blot, with stronger expression in brain and spleen than testis.

Identification of specific proteins on the surface of X- or Y-bearing sperm would be useful to develop an immunological technique for separating sperm. There is much indirect evidence regarding existence of a sex-specific protein [21,23,24] and many researchers have attempted to screen for SDM antigen on the Y sperm surface, then induced anti-SDM antibody for selection of females. However, antibodies prepared with male whole cells or cell lysates have typically failed to select female offspring [22,25], partly because male-specific proteins have similarity or near identity with

corresponding homologous proteins on the X chromosome [15]. As SDM antigen's candidate gene, *DBY* mRNA was detected in nearly 50% of mouse sperm. In the testis, it may be selectively reserved in sperm, and perhaps has an important role in DNA replication and transcript initiation during early embryonic development [26,27]. In addition, DBY is frequently absent in infertile patients [27], which could be related to impaired spermatogenesis. Expression of DBY protein was not detected in sperm by immunofluorescence and Western blot, although *DBY* transcripts were retained in capacitated mouse sperm [27]. Moreover, a DBY protein-specific antibody was not successfully prepared, due to high homology of DBY, Ddx3x and D1Pas1. In contrast, *Smcy* mRNA became undetectable in mouse sperm, although the SMCY (two epitopes of H-Y antigen) is expressed on the surface of sperm [9, 26]. More recently, rabbit-based polyclonal antiserum to peptides from Mea (peptide 1) or DBY (peptide 5) recognized nearly 50% of porcine sperm, whereas 95% of Y-bearing swine sperm had substantially reduced motility after being treated by these polyclonal antisera. Furthermore, the reduction was concentration-dependent; therefore, *Mea* and *DBY* might be candidate genes for SDM antigen in sperm [16]. Finally, DBY protein was detected on nearly 60% of mouse sperm (treated with DBY1-2-3 antibody). Therefore, we hypothesized that our antibodies recognized a specific epitope present in subpopulations of mouse sperm.

The SDM antigen is a complex encoded by a family of genes on the Y chromosome, or genes on other chromosomes, but their expression is regulated by genes on the Y chromosome (present only in males). Wang et al. reported technological improvements for construction of a recombinant Fab antibody against SDM antigen produced by phage display techniques [28]. Clearly there was a higher proportion of SDM-positive splenocytes in males versus females, although a minor proportion of female splenocytes were also SDM positive [28,29].

There is a clear need to further characterize this SDM antigen(s) and its (their) relevance for male cells. The DBY-2-3 antibody had stronger male and poorer female cells protein response, showed "non-specific" reaction, consistent with high expression in males than females of *Mea* as SDM candidate genes [30], may be explained by the minor interaction with those common components existing in DBY and DDX3X, D1Pas1. Regardless, its male specificity is noteworthy. In conclusion, the current DBY antibody against multi-epitope artificial antigen not only represented a candidate molecule for investigating male specific binding of DBY, it also has potential for isolation of X- and Y-bearing sperm.

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ISPITIVANJE IMUNOREAKTIVNOSTI VEŠTAČKI DOBIJENOG ANTITELA SPECIFIČNOG ZA MUŽJAKE KUNIĆA NA DBY MULTIEPITOPNI FUZIONI PROTEIN

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DEAD (Asp-Glu-Ala-Asp) box polipeptid 3, Y vezan (DBY ili Ddx3y) je gen kandidat za antigen specifičan za mužjake. DBY gen detektovan u mišijim spermatozoidima kodira predpostavljenu ATP- zavisnu RNK helikazu. Cilj je bio da se proizvede anti-predviđenu DBY multi-epitop fuziju antitela, što bi se moglo koristiti za determinaciju specifičnosti DBY za mužjake. Predviđanje epitopa je u cilju dizajniranja molekula koji mogu da imitiraju strukturu i funkciju epitopa, što predstavlja pomoć pri dizajniranju proteinskog molekula. Ova studija je predvidela DBY epitope, pripremila poliklonska antitela na DBY multi-epitop himerni protein i nakon toga ispitala njegovu imunoreaktivnost. Himerni protein koji je korišćen kao antigen se sastoji od tri DBY regiona sa najvećom divergencijom od ostalih članova porodice, skupno kloniranih (sa His obeležijem kako bi se olakšalo prečišćavanje). Nastalo antitelo je prepoznalo DBY 1-2-3 himerni protein i endogeno DBY protein iste veličine. Dalje, DBY protein je bio prisutan (Western blot) u testisu, splenocitima i mozgu mužjaka, dok je slabija traka bila uočena u mozgu i splenocitima ženke, a jajnik je proizvodio jedva uočljivu proteinsku traku. Optička gustina DBY proteina bila je viša kod mužjaka u poređenju sa odgovarajućim tkivima ženki. Na kraju, pozitivni signali DBY1-2-3 antitela bili su prisutni na svega ~60% zrelih mišijih speramatozoida (na osnovu imunofluorescencije i citometrije), u skladu sa očekivanom proporcijom Y- spermatozoida. Postavljena je hipoteza da antitela prepoznaju specifičan epitop prisutan u subpopulacijama spermatozoida miševa. Na osnovu toga zaključeno je da anti DBY 1-2-3 antitelo može biti alternativni put proizvodnje antitela na DBY protein. Štaviše, novo DBY antitelo protiv multi-epitopnog veštačkog antigena ima potencijal ne samo za ispitivanje specifičnog vezivanje DBY već i kao novi metod u selekciji pola.