Sexing murine embryos with an indirect immunofluorescence assay using phage antibody B9-Fab against SDM antigen

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ABSTRACT. The use of serologically detectable male (SDM; also called H-Y) antigens to identify male embryos may be limited by the source of anti-SDM antibody. In the present study, novel anti-SDM B9-Fab recombinant clones (obtained by chain shuffling of an A8 original clone) were used to detect SDM antigens on murine embryos. Murine morulae and blastocysts (n=138) were flushed from the oviducts of Kunming mice and incubated with anti-SDM B9-Fab for 30 min at 37°C. With an indirect immunofluorescence assay, the membrane and inner cell mass had bright green fluorescence (presumptive males). Overall, 43.5% (60/138) were classified as presumptive males and 56.5% (78/138) as presumptive females, with 85.0 and 88.5% of these, respectively, confirmed as correct predictions (based on PCR analysis of a male-specific [Sry] sequence). We concluded that the anti-SDM B9-Fab molecule had potential for non-invasive, technically simple immunological sexing of mammalian embryos.

KEYWORDS: embryo sexing, immunofluorescence, PCR, phage antibody, SDM antigen

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Although invasive methods, including PCR, cytogenetics and analysis of sex chromatin using Y-specific DNA probes (e.g. Sry gene), have been used for sex determination [4, 9], they require relatively advanced technical skills (e.g. breaching the zona pellucida to biopsy an embryo). Therefore, there is a clear need to develop a simpler method to identify male and female embryos without compromising accuracy.

Seralogically detectable male (SDM) antigen (also called H-Y, the Y-linked histocompatibility antigen) is a cell-surface glycoprotein complex in males [10, 14] that is expressed on embryos with more than eight cells [6, 10]. Detection of embryo SDM antigen by immunofluorescence and developmental arrest with SDM antibody have been used to determine embryonic sex in various mammals. However, poor affinity and male specificity of conventional antibodies limited their utility for embryo sexing [11, 14].

In previous studies [12, 13], a second SDM phage antibody library was constructed, and a high-affinity phage antibody clone B9 was isolated using affinity maturation by light- and heavy-chain shuffling of an original phage antibody clone A8 derived from B cells of mice immunized with male spleen cells. Using a cell-based ELISA, the B9 clone was positively selected by male splenocytes, whereas female splenocytes were negatively selected [13]. Furthermore, B9 had higher affinity than both the original A8 clone and conventional antibodies (SDM serum) [13]. The objective of the present study was to evaluate the presence of SDM antigen on murine embryos and sex murine embryos by an indirect immunofluorescence assay induced by B9-Fab. This phage antibody library was expected to provide new markers to investigate molecular mechanisms of SDM as a male-specific antigen for potential use in sex sorting of embryos in assisted reproduction.

The B9-Fab was prepared as reported [13]. The recombinant ph3-κ-Fd (light-and heavy-chain genes ligated into plasmid pComb3 vector) and DNA samples of B9 clone were transformed to E. coli XL1-Blue strain. Thereafter, expression of B9-Fab was induced by addition of IPTG (final concentration, 1.0 mmol/l), and B9-Fab soluble proteins were separated by centrifugation (10,000 × g for 15 min at 4°C). The quality of soluble Fab proteins in supernatant was assessed by non-reducing SDS-PAGE, followed by Coomassie Brilliant Blue staining, and was confirmed using horseradish peroxidase (HRP)-conjugated goat anti-mouse Fab (1:4,000, Pierce, Rockford, IL, U.S.A.) by western blot analysis, as described [13]. The end product B9 clone was selected for further analysis.

Morulae and blastocyst-stage embryos were recovered from superovulated Kunming female mice aged 8–10 weeks (SLAC Laboratory Animal Co., Ltd., ChangSha, China), as described [2]. Maintenance of mouse colonies and all experimental procedures were approved by the Animal Welfare and Ethics Committee of Hunan Agricultural University.

Thereafter, 5 to 8 murine embryos were placed in droplets (30 µl) containing B9-Fab (~8.5 µg/ml) diluted 1/3 (v/v) with RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubated at 37°C for 30 min. Embryos in control groups were incubated in droplets only with PBS. Embryos were washed three times with PBS and then placed in 30 µl droplets containing FITC-conjugated goat anti-mouse κ chain (Southern Biotech, Birmingham, AL, U.S.A.) diluted

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1/9 (v/v) with RPMI 1640 medium for signal detection. As a control for the background, only secondary antibodies were added, followed by three additional washes with PBS, and detection of fluorescence. When several cells of either the trophectoderm (TE) and/or the inner cell mass (ICM) displayed bright fluorescence, the embryo was deemed SDM positive (male), whereas if no cell-specific fluorescence was seen, the embryos were deemed SDM negative (female). A Chi-square test was used to determine any difference from an expected 1:1 sex ratio.
After fluorescence evaluation, the sex of each embryo was confirmed by multiplex PCR amplification of a murine male-specific sequence (Sry) and concurrent amplification of a non sex-specific IL3 gene (as a positive control). Each embryo was placed in 2 µl of lysis buffer (20 mM Tris-HCl, µl embryo was placed in 2 of a non sex-specific Il3 gene (as a positive control). Each male-specific sequence (Sry) and concurrent amplification was confirmed by multiplex PCR amplification of a murine controls, respectively) and distilled water (blank control) were included. Total 138 morula- and blastocyst-stage embryos were subjected to an indirect immunofluorescence assay. Furthermore, in our study, embryos were not different from a 1:1 ratio (P>0.05); genetic sex was confirmed by PCR.

Table 1. Detection of SDM antigen on murine morulae and blastocysts with B9-Fab

| Presumptive sex | No. (%) embryos at various stages | No. (%) embryos sexed by PCR |
|-----------------|----------------------------------|----------------------------|
| Male (SDM positive) |                                  |                            |
| Morula | 33♀ | 28 (84.8) | 5 (15.2) |
| Blastocyst | 27♂ | 23 (85.2) | 4 (14.8) |
| Total | 60 (43.5)♀ | 51 (85.0) | 9 (15.0) |
| Female (SDM negative) |                                  |                            |
| Morula | 41♀ | 5 (12.2) | 36 (87.8) |
| Blastocyst | 37♂ | 4 (10.8) | 33 (89.2) |
| Total | 78 (56.5)♀ | 9 (11.5) | 69 (88.5) |
| Total | 138 | 60 | 78 |

a) Proportions of fluorescent and non-fluorescent embryos were not different from a 1:1 ratio (P>0.05); genetic sex was confirmed by PCR.
embryos sexed using conventional SDM monoclonal and polyclonal antibodies prepared using male tissue or cells [1, 3]. Furthermore, there were no significant differences in the accuracy of sexing morulae (84.8% for males and 87.8% for females) versus blastocyst embryos (85.2% for males and 89.2% for females) with B9-Fab, which suggested that the immunological assay of SDM antigen was not affected by the stage of embryo development.

It was noteworthy that B9-Fab had roughly a 15% rate of misdiagnosis for both male and female embryos. Because embryo cells have complex surface components, with various membrane proteins being exposed, we concluded that minor interactions (fluorescence-positive) between non-SDM surface antigens of embryo cells or common components on the cell surface of both sexes, and B9-Fab were observable, which presumably contributed to misdiagnosis in sexing embryos. Lower-quality murine embryos with fluorescence unrelated to presence of SDM antigen might also cause misdiagnosis. However, unlike conventional antibodies against the SDM antigen, SDM B9 Fab fragments could be engineered genetically to enhance specificity and/or binding activity. In that regard, the amino acid sequence in the high and light chains of B9 Fab could be modified (mutagenesis strategy or combining a bright fluorescent protein with B9 Fab) to improve accuracy. Therefore, although the assay was reasonably accurate, targeted modifications and the effects of those molecular changes on the accuracy of this approach for sexing embryos should be explored in future studies.

In conclusion, the present study confirmed previous reports of the efficiency of sexing early embryos using an antibody against SDM antigen. Notwithstanding, this was apparently the first report of sexing of murine embryos by an indirect immunofluorescence assay induced by novel phage antibody, highlighting potential application of SDM antibodies in assisted reproduction technology.

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