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Sex Determination of Bovine Embryos Using H-Y Antibodies

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Avery, B. and M. Schmidt: Sex determination of bovine embryos using H-Y antibodies. Acta vet. scand. 1989, 30, 155–164. – 6 days old bovine embryos (n = 126) were obtained from 8 superovulated cows or heifers by flushing the uteri and oviducts either non-surgially or after slaughter. Part of the embryos (n = 72) (morula stages) were placed in Ham's F-10 or PBS supplemented with 10 % fetal calf serum (FCS) diluted 1:1 with supernatant from the H-Y antibody producing clone and cultured at 38°C, in 5 % CO_2/95 % air and 100 % humidity. Control embryos (n = 54) were cultured in H-Y antibody free medium.
After culture the embryos could be separated into a blastocyst- and a morula group. A subsequent colchemid and hypotonic treatment and fixation and Giemsa staining allowed a precise karyotyping, and thus sex determination for 36 H-Y antibody treated embryos and 22 control embryos. The limiting factor for proper karyotyping was lack of metaphases, incomplete methaphases or poor preparation. Among the H-Y antibody treated embryos we found 7 males and 15 females in the blastocyst and 14 males and 0 females in the morula group. A statistical analysis of these proportions led to the conclusion that the H-Y antibody had a significant influence on the sex ratio.

sex ratio; cattle; karyotyping; monoclonal antibodies.

Introduction

The H-Y antigen was first described by *Eichwald & Silmser* in 1955 as a male-specifik weak transplantation antigen system. It is found in the cell membrane of almost all nucleated male mammalian cells and appears to be highly conserved throughout evolution, as mouse H-Y antibodies are known to cross react with H-Y antigen from seventy different species (*Wachtel et al.* 1974, 1975, *Wachtel* 1983, *Silvers & Wachtel* 1977, *Nakamura et al.* 1984).

It has been questioned whether the male specific antigen which can be detected serologically is the same as the originally described transplantation antigen (*Melvold et al.* 1977, *Wachtel et al.* 1984), but for all practical purposes they are considered to be identical and hence the terminology H-Y antigen is used.

In addition to being detected on adult somatic nucleated cells and sperm (*Bennett & Boyse* 1973, *Goldberg et al.* 1971, *Hoppe & Koo* 1984, *Koo et al.* 1973, *Zaborski* 1979) H-Y antigen was also found on 8-cell mouse embryos by *Krco & Goldberg* (1976). They observed that half of the mouse embryos died when incubated with H-Y antibody and complement. Later *Epstein et al.* (1980) were able to show by karyotyping that 92 % of the surviving embryos were females. Besides being found on mouse embryos, H-Y antigen has been demonstrated on embryos from other mammalian species: rat, rabbit,

pig, sheep, cow, goat and horse. This lack of species specificity combined with the presence of a H-Y antigen on male embryos has made mouse H-Y antibody of potential use in the embryo transfer industry as a mean to control sex ratio, although it is still at the experimental stage (reviewed by *Anderson* (1987) and *Wachtel* (1984)).

Until now sexing of embryos by means of H-Y antibodies has been attempted by the following 3 methods:

a) Incubation with H-Y antibody and complement, which leads to destruction of the male embryos (*White et al.* 1982, 1983, *Wachtel* 1984, *Shelton & Goldberg* 1984).
b) Incubation with H-Y antibody followed by FITC-labelled goat-anti-mouse-immunoglobulin. The FITC-labelling allow visualization of male embryos and full viability of both sexes (*Wachtel* 1984, *White et al.* 1983, 1984, 1987).
c) Incubation in H-Y antibody containing medium for some hours, after which a normally developing blastocyst group (females) and a reversibly arrested morula group (males), both viable, can be separated (*Utsumi et al.* 1983, 1984).

The last method was based on the observation that after culturing rat morulas in H-Y antibody containing medium, the development of half of the embryos was temporarily arrested. After being transferred to recipients, the male ratio from the blastocysts was 20 %. The same effect was seen in cow and goat morulas (*Utsumi et al.* 1983, 1984). This non-invasive and non-destructive approach for sexing cattle embryos seemed very attractive, and the present experiment was undertaken to test the efficiency of our H-Y antibody on bovine embryos using the *Utsumi method.*

Materials and methods

Embryo source

Eight to 14 days after estrus dairy cows and heifers were stimulated with either a single injection of 1500–3000 I U. PMSG[a] (n = 5) or injections of 32–40 mg FSH[b] (n = 4) given twice daily in decreasing doses for 5 days. 72 h after the initial injection the animals were given 0.75 mg cloprostenol[c] to cause luteal regression. Insemination occurred at 12 and 24 hours after onset of heat, and at day 6 the uteri were flushed non-surgically or surgically after slaughter. The flushing medium was Dulbecco's phosphate-buffered saline (PBS).

Embryo evaluation

Morphological evaluation was based on developmental stages and quality (*Lindner & Wright* 1983):

[a] Antex(R), Leo Pharmaceuticals, Ballerup, Denmark.
[b] FHS-P, Burns Biotec, Omaha, Nebraska, USA.
[c] Estrumat(R), Vet, Lundbeck and Co. Valby, Denmark.

Developmental stages

Age	Stage	Description
day 5	morula (M)	32–64 cells in a ball; the cellular mass occupies almost all of the perivitelline space
day 6	compact morula (CM)	64 cells which have "fused" into a compact mass of homogenous structure. The cell mass occupies 60–70 % of the perivitelline space.
day 6-7	young blastocyst (YB)	The embryo begins to form blastocoele which is a fluid filled cavity. The embryo occupies up to 70–80 % of the perivitelline space, and up to 50 % is blastocoele. (Signet ring).
day 7	blastocyst (BL)	The embryo occupies 80–100 % of the perivitelline space, and the blastocoelom occupies more than 50 % of the embryo.
day 8	expanded blastocyst (XB)	The diameter increases 1.5 times, and as a result the zona pellucida becomes very thin. The inner cell mass is small and compact.
day 9	hatched blastocyst (HA)	The hatching process takes 8–12 hours, where the zona pellucida is being shed.

Quality. The quality was based on morphology divided into 4 grades: excellent (A), good (B), fair (C), poor (D).

Embryoculture

Embryos were cultured in Hams F-10 medium (Flow), supplemented with glutamin 4 mM, penicillin 200 IE/ml, streptomycinsulphate 100 µg/ml and 10 % fetal calf serum (FCS) (Seralab), or in Dulbeccos PBS medium (from our own pharmacy) containing glucose 1000 mg/l, pyruvate 36 mg/l, kanamycinsulphate 25 mg/l, BSA 4 g/l supplemented with 10 % FCS or 10 % steer serum, in Nunc 4-well dishes at 38°C in 5 % Co_2/95 % air and 100 % humidity.

Supernatant from the H-Y antibody producing clone, cultured in RPMI-1640 (Flow) supplemented with 10 % FCS provided the H-Y antibody, which was diluted 1:1 with the embryo culture medium.

Karyotyping and sex determination

These procedures were similar to those described previously (*Avery et al.* 1989).

Production of monoclonal H-Y antibody

Serum from multiparous female BALB/c mice (8–12 litters) was tested for the presence of H-Y antibody by a cytotoxicity assay against sperm (*Krupen-Brown & Wachtel* 1979, *Piedrahita & Anderson* 1985, *Dooher & Bennett* 1977, *Goldberg et al.* 1971). Three days before fusion, 5×10^7 male spleen cells from the same strain was given intraperitoneally to one of the H-Y antibody positive multiparous mice. The spleen cells from the female mouse were fused with cells from the BALB/c mouse myeloma cell line P3-X63-Ag8-U1 (PU) in the presence of PEG using standard techniques (*Köhler & Milstein* 1975, *Kenneth & McKearn* 1980, *Gefter et al.* 1977, *Goding* 1980, *Yelton et al.* 1978).

The hybridoma supernatants were tested for H-Y antibody activity by an ELISA technique on living confluent monolayers of primary epidermal cells from newborn female and male Wistar rats. One clone was found to react with male but not with female cells. The hybridoma supernatants were also tes-

ted on Daudi cell supernatant coated onto ELISA plates. (Daudi cells, ATCC no. CCL213). (*Bradley & Heslop* 1984, *Brunner et al.* 1984, *Meck et al.* 1984, *Nagamine et al.* 1984). The same clone was found to react. Daudi cells are beta-2 microglobulin deficient male B-lymphoblasts, which are secreting H-Y antigen to the surrounding medium. Cells from the clone producing H-Y antibodies was subcloned by limiting dilution, and supernatant from this clone, which is still producing IgM antibodies was used in the present study.

Experiments and results

Eight donors were flushed at day 6, i.e. at the expected day for the embryos being at the morula stage, yielding 126 embryos. Thirty five percent (n = 44) of the embryos were quality A, 46 % (n = 58) and 19 % (n = 24) were quality B and C, respectively. Five different experiments were set up, and the embryos were divided into a H-Y antibody group (A) (n = 72) and a control group (B) (n = 54). The control embryos were cultured under identical conditions in H-Y antibody-free medium. It was attempted to allocate the embryos in such a way that embryo number, -quality and -stages were comparable for group A and B. The distribution of the developmental stages and the allocation into the 5 experiments is seen in Table 1.

During culture the embryos were evaluated every second hour. When approximately half of the H-Y antibody treated embryos had developed into blastocysts, they were

Table 1. Embryo development of group A and B embryos

			Blastocysts		Compact morulas	
Exp	Group	Initial embryo development	development	hours in culture	development	hours in culture
1	A	8 CM	2 BL	17	6 CM	17
	B	1 BL, 2 YB	1 XB, 2 BL	17	0	
2	Á	9 CM	4 BL	15	5 CM	19
	B	8 CM	5 BL	15	3 CM	19
3	A	20 CM	11 BL	11	9 CM	14
	B	20 CM	13 BL	10	7 CM	10
4	A	19 CM	12 BL	10	7 CM	13
	B	19 CM	15 BL	10	4 CM	10
5	A	16 CM	10 BL	4	6 CM	4
	B	4 YB	4 BL	4	0	
Total	A	72	39 BL		33 CM	
	B	54	40 BL		14 CM	

A: embryos cultured in Hams F-10/10 % FCS (exp. 1 and 2), in PBS/10 % FCS (exp. 3 and 4) or in PBS/10 % steer serum (exp. 5) diluted 1:1 in super natant from the H-Y antibody clone. Following culture two groups were distinguished: blastocysts and compact morulas.

B: control embryos cultured under identical conditions in H-Y antibody free medium.

divided into 2 groups (BL and CM) and transferred to fresh H-Y antibody free medium. The BL were colchemid treated and karyotyped immediately after, the CM were cultured for 0–4 h longer before colchemid treatment and karyotyping. The control embryos were cultured and karyotyped parallel to the H-Y antibody treated embryos.

Table 1 also shows, that following culture for group A embryos in H-Y antibody containing medium, 39 embryos (54 %) developed to blastocysts, while 33 embryos (46 %) remained at the compact morula stage. In the control group (B), 40 embryos (74 %) developed into blastocysts, and 14 (26 %) embryos stayed at the compact morula stage at the end of the experiment. This difference was statistically significant (chi square = 5.229, p = 0.022, DF = 1).

The outcome of the karyotyping is shown in Table 2. In the H-Y antibody treated group of embryos (A), 7 males and 15 females versus 14 males and 0 females were found among the blastocysts and compact morulas, respectively. In group A, embryo sex and embryo stage was significantly correlated, with blastocysts predominantly being females and compact morulas being males (chi

Table 2-1. Embryo development and karyotyping of the H-Y antibody treated embryos (Group A).

	Blastocysts				Compact morulas			
		typed				typed		
Exp	no embryos	male	female	not typed	no embryos	male	female	not typed
1	2	0	1	1	6	3	0	3
2	4	0	1	3	5	1	0	4
3	11	2	4	5	9	4	0	5
4	12	3	5	4	7	4	0	3
5	10	2	4	4	6	2	0	4
Total	39	7	15	17	33	14	0	19

Table 2-2. Embryo development and karyotyping of the control embryos (Group B).

	Blastocysts				Compact morulas			
		typed				typed		
Exp	no embryos	male	female	not typed	no embryos	male	female	not typed
1	3	0	2	1	0	0	0	0
2	5	3	0	2	5	0	0	3
3	13	1	3	9	7	1	3	3
4	15	4	4	9	4	0	1	3
5	4	2	0	2	0	0	0	0
Total	40	8	9	23	14	1	4	9

Table 3. Total number of embryos which could be karyotyped in the H-Y antibody (A) and the control group (B).

		No embryos					
Group	no embryos	not typed	typed	male	female	% typed	sex ratio %
A	72	36	36	21	15	50	58
B	54	32	22	9	13	41	41
Total	126	68	58	30	28	45	52

square = 16.364, p = 0.000, DF = 1). In the control group (B), 8 males and 9 females versus 1 male and 4 females were found in the blastocyst and compact morula groups, respectively. This difference was not statistically different (chi square = 1.170, p = 0.279, DF = 1).

Only 40–50 % of the embryos could be karyotyped. The proportion of males for the total group of embryos was 52 % with 58 % in group A and 41 % in group B embryos. This difference was not significant (chi square = 1.660, p = 0.198, DF = 1).

The limiting factors for proper karyotyping were lack of metaphases (17 %), poor preparation (25 %) and gross chromosomal abnormality (12 %); the latter was found among embryos of quality c (n = 24, 3 haploids, 1 triploid, 2 tetraploids and 2 polyploids).

Embryos which could not be karyotyped were omitted from the statistics, since their developmental stages were distributed not significantly different from that of the typed embryos. (A embryos: chi square = 1.399, p = 0.237; B embryos: chi square = 0.198, p = 0.657).

The number of experimental (A) and control (B) embryos that could be karyotyped did not differ significantly from each other either (chi square = 1.619, p = 0.203, DF = 1).

In addition to these 5 experiments a 6th experiment was performed, where 6 H-Y antibody treated embryos of excellent quality from one donor were transferred to 6 recipients. The embryos were flushed non-surgically. After 13 h in culture 2 groups could be distinguished: 3 BL and 3 CM. After wash in H-Y antibody free medium, they were transferred separately to 6 recipients.

The outcome of the 6th experiment was not conclusive. Three cows did not become pregnant, 2 had prolonged cycles (day 29 and day 50) and one gave birth to a male calf, resulting from a compact morula.

Discussion

The method described in this paper was based on culture in H-Y antibody containing medium followed by karyotyping of whole embryos. The purpose was to test if our H-Y antibody could discriminate between male and female embryos. The results presented demonstrate that early embryonic development and thus the sex of bovine embryos was influenced by the antibody. These observations are in accordance with findings by other groups (*King* 1984, *Hare et al.* 1976, *Picard et al.* 1984, *Rall & Leibo* 1987, Singh & Hare 1980, *Winterberger-Torres & Popescu* 1980).

In our 6th experiment the pregnancy rate was lower than expected, since only 1 em-

bryo of 6 resulted in offspring. It is very likely that prolonged cell culture contributed to this decrease in embryo viability, as described by *Renard et al.* (1980a, b).

In agreement with *Utsumi* (1983, 1984) we emphasize that this method requires embryos at the compact morula stage. In our initial experiments we cultured mouse embryos from the 4–8 cell stages in H-Y antibody with no effect on their development until they reached the compact morula stage. Then their development deviated, and it was possible to find a blastocyst versus a compact morula group. Later stages i.e., blastocysts were equally unaffected by the H-Y antibody treatment.

By means of immunofluorescence H-Y antigen has been detected on mouse embryos from the 8-cell stages, but not on the 4-cell stages. It is easily detected on 8-cell mouse embryos and on morulas, but is more difficult to detect on blastocysts (*Wachtel* 1984, *Anderson* 1987).

White et al. (1987) were able to demonstrate fluorescence on the inner cell mass, but not on trophectoderm cells from bovine expanded blastocysts, proposing that this distribution of H-Y antigen could be protecting the implanting embryo from rejection by the maternal organism.

H-2 antigens, which are major histocompatibility antigens in the mouse (MHC), are expressed on mouse embryos. *Goldbard et al.* (1985) showed by means of monoclonal anti-H-2-antibody that all stages from unfertilized eggs to blastocysts synthesize H-2 antigens, but it becomes increasingly difficult to demonstrate on blastocysts, and it is generally believed that the antibody is unable to penetrate to the blastocele, so only H-2 antigens on the outside of the blastocysts are detected.

The H-Y antigen system, although not a MHC could act in the same way and this would explain its inability to control blastocyst development. It is unclear though why the development of male embryos is retarded in H-Y antibody containing medium, but a possible explanation could be capping and internalization of the H-Y antigen/H-Y antibody complex on the male embryos, an energy requiring reaction, which might delay development.

Another explanation could be that H-Y antigen could act as a male growth regulator as stated by *Mittwoch* (1977), and that H-Y antibody could inhibit this regulation.

The most commonly used sexing method, based on H-Y antibodies is the indirect immunofluorescent method, where the male embryos after a brief incubation (30 min) in H-Y antibody are labelled with FITC-labelled-goat-anti-mouse-immunoglobulin. This method is fast but requires an inverted fluorescence microscope. The sorting and evaluation of embryos is highly subjective and a considerable experience is needed in order to avoid false positive reactions. Embryo viability has by some groups been reported unaffected, while others find it decreased (*White et al.* 1985, 1987, *Anderson* 1987).

The major advantages of the *Utsumi* method i.e. the method described in this study are independence of elaborate equipment and applicability on porcine embryos (*Avery* 1987). Due to the high number of sperm in the zona of fertilized porcine embryos, the fluorescence method is not feasible, but requires removal of the zona before FITC-labelling. The disadvantages of the Utsumi method are relatively long incubation periods, having negative effect on embryo viability, and suitable embryo stages strictly limited to compact morulas.

Conclusion

The H-Y antibody used in these experiments seems to be able to distinguish between male and female bovine embryos with the same accuracy as described by other authors.

The use of H-Y antibodies is only one of the possible methods available for preimplantatory sexing, but none of the methods are at the moment suitable "in the field". At present we are investigating alternative methods, especially the possible correlation between early embryonic development and sex.

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Sammendrag

Kønsbestemmelse af bovine embryoner ved hjælp af H-Y antistoffer

Seks dage gamle embryoner (n = 126) blev opsamlet fra 8 superovulerede køer eller kvier, enten ved ikke-kirurgisk skylning eller ved skylning efter slagtning. Embryonerne, som var på morulastadiet, blev delt i en forsøgsgruppe (n = 72) og en kontrolgruppe (n = 54), som var sammenlignelige med hensyn til kvalitet og udviklingsstadier, bedømt ved morfologiske kriterier.

Embryonerne blev enten dyrket i Hams F-10 medium eller i Dulbeccos PBS tilsat 10 % føtalt kalveserum (FCS), ved 38°C i 5 % CO_2/95 % atmosfærisk luft og 100 % fugtighed. Forsøgsgruppens embryoner blev dyrket i et af de ovennævnte medier fortyndet 1:1 med supernatant fra det monoklonale H-Y antistof. Efter dyrkning i ca. 10 timer havde 54 % (39) af embryonerne nået blastocyststadiet, mens 46 % (33) forblev på morulastadiet. I kontrolgruppen sås 74 % (40) blastocyster og 26 % (14) morulae.

Karyotypning med kønsbestemmelse kunne udføres for 36 af de H-Y antistof behandlede embryoner (50 %). I blastocystgruppen fandtes 7 hanner og 15 hunner, og i morula gruppen var der 14 hanner og 0 hunner. Kønsforskellen mellem de to grupper var statistisk signifikant. I kontrolgruppen kunne 22 embryoner karyotypes (41 %) med 8 hanner og 9 hunner i blastocystgruppen og 1 han og 4 hunner i morulagruppen. Disse fordelinger afveg ikke fra den forventede 1:1 fordeling.

Begrænsende for korrekt karyotypning var manglende og/eller inkomplette metafaser eller præparater hvor detaljer ikke kunne skelnes.

Det konkluderes, at H-Y antistoffet kan udøve en effekt på kønsfordelingen, når det anvendes på morulastadier, ved at hæmme hanembryonernes udvikling reversibelt, så de forbliver på morulastadiet, mens hunembryonerne ikke påvirkes, men udvikles videre til blastocyststadiet.

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