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Amélie Bonnet-Garnier, S. Lacaze, J.F. Beckers, Hélène Berland, Alain Pinton, Martine M. Yerle, Alain Ducos

**Original Article**

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# Meiotic segregation analysis in cows carrying the t(1;29) Robertsonian translocation

A. Bonnet-Garnier[a] S. Lacaze[b] J.F. Beckers[c] H.M. Berland[a] A. Pinton[a] M. Yerle[a] A. Ducos[a]

[a]UMR 444 INRA-ENVT, Génétique cellulaire, Toulouse, [b]MIDATEST, Soual (France); [c]Département de physiologie animale, Université de Liège, Liège (Belgium)

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**Abstract.** Heterozygous carriers of Robertsonian translocations generally have a normal phenotype but present reproductive failure. In cattle, the t(1;29) Robertsonian translocation is very common and carriers show a 3–5% decrease in fertility. Some data suggest that female carriers have a higher decrease than male carriers but no direct studies of the chromosome content of oocytes from a t(1;29) carrier cow have been performed so far.

Four heterozygous carrier cows underwent hormonal stimulations and follicles punctions and about 800 oocytes were matured in vitro. Six hundred metaphase II preparations were obtained and analysed by fluorescent in situ hybridization with bovine chromosome 1 and 29 painting probes. Proportions of different kinds of oocytes were assessed: 74.11% (292/394) were normal and balanced, 4.06% (16/394) unbalanced and 21.83% (86/394) diploid. For all cows, the number of normal oocytes was not significantly different from the number of translocated oocytes but the diploidy and unbalanced rate were significantly different between them. As found in bulls, the meiotic segregation pattern in cows has shown a preponderance of alternate products. However, the frequency of unbalanced gametes determined in females (4.06%) was significantly higher than the frequency observed in males (2.76%). The divergence in the rate of diploid gametes (0.04% vs. 21.83%) is mainly explained by the difference between males and females.



Robertsonian translocations are the most frequent structural chromosomal abnormalities observed in humans (Nielsen and Wohlert, 1991) and cattle (Fries and Popescu, 1999). Heterozygous carriers of Robertsonian translocations generally have a normal phenotype but show variable decreases in fertility (Roux et al., 2005). Indeed, these carriers can produce a significant percentage of unbalanced gametes which lead to recurrent spontaneous abortions (Munné et al., 1998a; Roux et al., 2005).

Since 1995, the meiotic segregation patterns of Robertsonian translocations have been particularly studied in men using the sperm-FISH technique which allows examination of a large amount of spermatozoa (several thousands) per patient (Anton et al., 2007). These studies (12 publications for 51 patients, reviewed in Anton et al., 2007) have found a majority of normal or balanced spermatozoa (ranging from 60 to 93%).

For obvious ethical reasons, such direct studies are very difficult to perform in women. Female meiotic segregation is generally ascertained by analysis of the first polar body or embryo biopsy in the case of preimplantation genetic diagnosis (Munné et al., 1998b, 2000; Durban et al., 2001; Pujol et al., 2003). From such studies, estimated rates of unbalanced gametes were generally higher for females than for males but the number of oocytes analyzed so far remains limited (from 30 to 100 of several women, Munné et al., 2000; Durban et al., 2001; Pujol et al., 2003).

Request reprints from A. Bonnet-Garnier
UMR444 INRA-ENVT, Génétique Cellulaire
23 Chemin des capelles, BP 87614
FR–31076 Toulouse Cedex 3 (France)
telephone: +33 1 61 19 39 22; fax: +33 1 61 19 39 24
e-mail: a.garnier-bonnet@envt.fr

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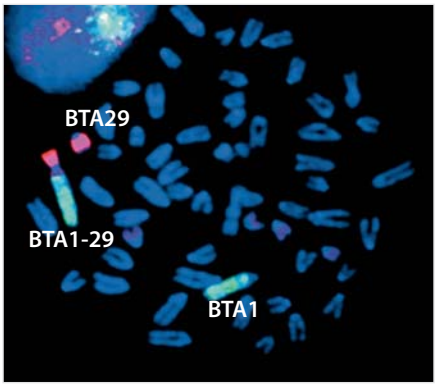


**Fig. 1.** Hybridization of bovine chromosomes BTA1 (green) and BTA29 (red) commercial painting probes on the metaphase of a t(1;29) heterozygote carrier.

In cattle, the occurrence of the well known t(1;29) offers a model to study the chromosome segregation in female carriers of a Robertsonian translocation. Indeed, the t(1;29) Robertsonian translocation is the most widespread chromosomal abnormality in cattle breeds (Fries and Popescu, 1999). In France, the Blonde d'Aquitaine breed shows the highest incidence of this translocation with 8.5% of heterozygous carriers observed within males submitted to chromosomal control.

Previous studies (Gustavsson, 1969, 1971; Refsdal, 1976; Dyrendahl and Gustavsson, 1979) demonstrated the negative effect (3–5%) of this translocation on reproductive performance of both male and female heterozygotes. By using the sperm-FISH technique our recent study revealed that, on average, 97.1% of spermatozoa were normal or balanced in the semen of t(1;29) carrier bulls (Bonnet-Garnier et al., 2006). Nevertheless, some data suggest that the percentage of unbalanced gametes is higher in cows than in bulls heterozygous for the t(1;29). Indeed, fertility criteria (calving intervals, non-return to service rate, culling rate) are more depressed for the t(1;29) carrier cows than for the mates of carrier bulls (Refsdal, 1976; Dyrendahl and Gustavsson, 1979; Maurer and Vogt, 1988). Moreover, the study of Schmutz et al. (1991) indicated that the percentage of unbalanced embryos is higher if they are produced by carrier cows (36%) than by carrier bulls (19%). Although the number of embryos studied is reduced, they concluded that the translocation has a greater negative effect on females than on males.

In cattle, reproductive technologies (hormonal superstimulation and transvaginal oocyte aspiration, Pieterse et al., 1991; in vitro maturation and fertilization, Brackett, 1983; Kruip et al., 1994; Lacaze et al., 1997) are commonly used by the breeding organizations to obtain a large number of oocytes and then embryos (Galli et al., 2001). We have used this technology to collect immature oocytes from several t(1;29) carrier cows. The oocytes of each cow were matured in vitro (Brackett, 1983) separately and treated in order to determine their meiotic stage and content (King et al., 1986; Sosnowski et al., 1996). The aim of our project was thus (i) to estimate the rates of unbalanced and balanced oocytes in four cows by fluorescence in situ hybridization (FISH) on metaphase II oocytes and polar bodies, (ii) to examine a possible inter-individual variation of these rates and (iii) to compare the results with those obtained previously for bull carriers of the same chromosomal rearrangement (Bonnet-Garnier et al., 2006).

## Material and methods

*Animals, oocyte recovery and in vitro maturation*

Four healthy, non-pregnant Blonde d'Aquitaine cows (identified by the numbers 1, 2, 3 and 4) from five to ten years of age were used. All animals received an ear implant (Crestar®, Intervet, Belgium: implant norgestomet 3 mg). The cows were superstimulated with injections of pFSH (Stimulfol®, FMV Liège, Belgium) and subjected to ovum pick-up (OPU) 21 times at two-week intervals over a period of one year. Follicles, between 5 and 16 mm of size, were aspirated with a needle under transvaginal ultrasound guidance. The oocytes of each cow were cultured in vitro separately in 4-well dishes containing 500 μl of maturation medium (TCM199 medium without Hepes supplemented with 15% fetal calf serum, 10 μg/ml of FSH, 1 μg/ml of estradiol-17β and 10 ng/ml of EGF) for 22–27 h. All cultures were incubated at 39°C in a humidified atmosphere of 5% $CO_2$ and air.

*Metaphase II oocyte preparations*

After at least 22 h of incubation at 39°C, the cumulus and the corona radiata cells were removed mechanically by pipetting. A modified method of Tarkowski (1966) was used for metaphase II oocytes preparation. Briefly, denuded oocytes were incubated in a hypotonic solution (1% sodium citrate) at 39°C for 8–10 min, then placed on a superfrost plus® slide. Small droplets of cold fixative (ethanol:acetic acid 3:1) were added carefully onto the oocyte until the zona pellucida disrupted, the cytoplasm lysed and the chromosomes spread. Then each slide was examined under a phase contrast microscope in order to check for the presence of metaphase spreads and stored at –20°C until used for the FISH procedure.

*Fluorescence in situ hybridization and detection of the probes*

Hybridizations were carried out as described by Yerle et al. (1994) and Hayes et al. (1992) with some modifications. Briefly, before hybridization, the slides were incubated at 37°C for 45 min in RNase (100 μg/ml, Sigma-Aldrich, France) followed by a pepsin treatment (Sigma-Aldrich) for 5 min (pH 3, 20 μg/ml) and dehydrated in ethanol (70%, 80%, 100%). The denaturation was performed in 70% formamide, 2× SSC (pH 7) for 2 min exactly at 76°C. Commercial whole chromosome painting probes for bovine chromosomes 1 and 29 (BTA1 and BTA29) were used according to the manufacturer's protocol (Cambio, UK) with minor modifications. The probes were tested for hybridization on normal bovine metaphases (see Fig. 1) as previously described in Bonnet-Garnier et al. (2006). After few minutes at 37°C, they were denatured at 76°C for 10 min, incubated at 37°C for 1 h to pre-anneal repeated sequences and hybridized to the slides in a dark moist chamber at 37°C for 48 h. Afterwards, the slides were washed three times in 50% formamide, 2× SSC (pH 7) for 3 min at 45°C and four times in 2× SSC (pH 7) for 3 min at 45°C. Hybridization signals were revealed as described in Pinton et al. (2004). BTA1 was directly labelled with fluorescein and BTA29 labelled with biotin was detected using streptavidin coupled to Alexa 594 (Molecular Probe, Invitrogene SARL, France). The slides were counterstained with DAPI in antifade (Vectashield® Vector, UK) and observed under a fluorescence microscope (Zeiss Axioskop) fitted with a triple bandpass filter. The images were recorded with the CytoVision® software (Applied Imaging, USA). The oocytes were included in the analysis in case they displayed unambiguous FISH signals and a complete metaphase at the same time, or an incomplete metaphase but an intact first polar body or in few cases the first polar body alone.

**Table 1.** Distribution of the products of segregation modes for male and female heterozygous carriers of the Robertsonian translocation t(1;29)

| Segregation mode | Chromosomal constitution | In situ fluorescent phenotype | Cow gametes % (number of cells) | Cows, corrected rate (without diploids) in % | Bull gametes % (number of cells)[a] | Bulls, corrected rate (without diploids) in % |
|---|---|---|---|---|---|---|
| Alternate | 1q/29q or der(1q;29q) | Normal | 74.11 (292) | 94.81 | 97.21 (7900) | 97.21 |
| Adjacent | 29q | Nullisomy 1 | 1.01 (4) | 1.30 | 1.36 (111) | 1.36 |
| Adjacent | 1q/der(1q;29q) | Disomy 1 | 1.01 (4) | 1.30[b] | 0.11 (9) | 0.11 |
| Adjacent | 1q | Nullisomy 29 | 0.52 (2) | 0.65 | 0.80 (65) | 0.80 |
| Adjacent | 29q/der(1q;29q) | Disomy 29 | 0.76 (3) | 0.97 | 0.48 (39) | 0.48 |
| Total of adjacent products | | | 3.30 (13) | 4.22 | 2.76 (224) | 2.76 |
| PSSC[c] | | | 0.76 (3) | 0.97 | | |
| Total of unbalanced products | | | 4.06 (16) | 5.19[d] | 2.76 (224) | 2.76 |
| 3:0 or diploidy | 1q/29q/der(1q;29q) or 1q/1q/29q/29q | Diploidy | 21.83 (86)[b] | | 0.04 (3) | |
| Total number of gametes scored | | | 394 | 308 | 8,127 | 8,124 |

[a] Results published in Bonnet-Garnier et al. (2006).
[b] $P < 0.001$.
[c] Premature separation of sister chromatids.
[d] $P < 0.05$.

*Statistical analysis*

A classical $2 \times 2$ $\chi^2$ test with the Yates correction for continuity was used to compare the proportions of unbalanced gametes between females and males (Dagnelie, 1975). Differences were considered to be significant if $P < 0.05$.

Contingency table with a Pearson $\chi^2$ was performed with R freeware to compare proportions of diploid, balanced and unbalanced gametes between females (R project, 2005 http://www.R-project.org).

## Results

*Oocyte collections and chromosome analysis*

A total of 850 follicles were punctured (of 4 cows) and 770 of the 817 oocytes recovered were matured in vitro. The average number of oocytes recovered per cow during the 21 OPU sessions ranged from 5.9 to 13.9. Among the 597 oocytes successfully spread and used for FISH analysis, 394 (66%) produced convenient results. Out of these 394 oocytes, 292 (74.11%) were balanced including 152 'translocated' and 140 'normal' cells, 86 (21.83%) were diploid and 16 (4.06%) were unbalanced. The proportions of segregation products are presented in details in Table 1 and illustrations of the different cases of oocytes observed are depicted in Fig. 2.

The number of 'translocated-balanced' oocytes (Fig. 2a) is slightly higher than the number of normal ones (Fig. 2b) but this difference is not statistically significant (Table 2). There is no significant departure from 1:1 ratios for the adjacent categories: nullisomy 29 (Fig. 2c) vs. disomy 29 (Fig. 2f) or nullisomy 1 (Fig. 2d) vs. disomy 1 (Fig. 2e). Three cases (0.76%) of premature unbalanced separation of sister chromatids (PSSC) were found which correspond in all cases to oocytes with the translocated chromosome and an extra chromatid of BTA1 (Fig. 2h).

**Table 2.** Distribution of normal and 'translocated-balanced' oocytes in the four cows

| Animal | BTA1 and BTA29 | t(1;29) | Total of oocytes analyzed | *P* value |
|---|---|---|---|---|
| Cow No. 1 | 54 | 57 | 135 | 0.805 |
| Cow No. 2 | 37 | 32 | 78 | 0.519 |
| Cow No. 3 | 20 | 28 | 59 | 0.186 |
| Cow No. 4 | 29 | 35 | 122 | 0.467 |
| Total | 140 | 152 | 394 | 0.417 |

*Comparison of the segregation profiles between cows*

Table 3 presents in details the proportions of balanced, unbalanced and diploid oocytes assessed for the four cows. The two older cows (No. 2 and No. 3) gave less oocytes (78 and 59) than the two younger (135 and 122). On the other hand, the rates of diploid (from 7.7 to 45.1%) and the corrected rate of unbalanced oocytes (from 2 to 7.5%) are statistically different between cows ($P < 0.0001$). However, in the case of unbalanced oocytes, the results of the $\chi^2$ test should be considered with caution because the number of oocytes is too small (<5 for three of the four cows).

*Comparison between females and males*

The percentage of normal and balanced gametes (74.11%) in females is lower than the proportion of normal or balanced spermatozoa (97.21%) observed for two heterozygous bulls (Table 1). This difference is primarily due to the higher rate of diploid gametes in females (21.83%) compared to males (0.04%). If diploids are not taken into account, the

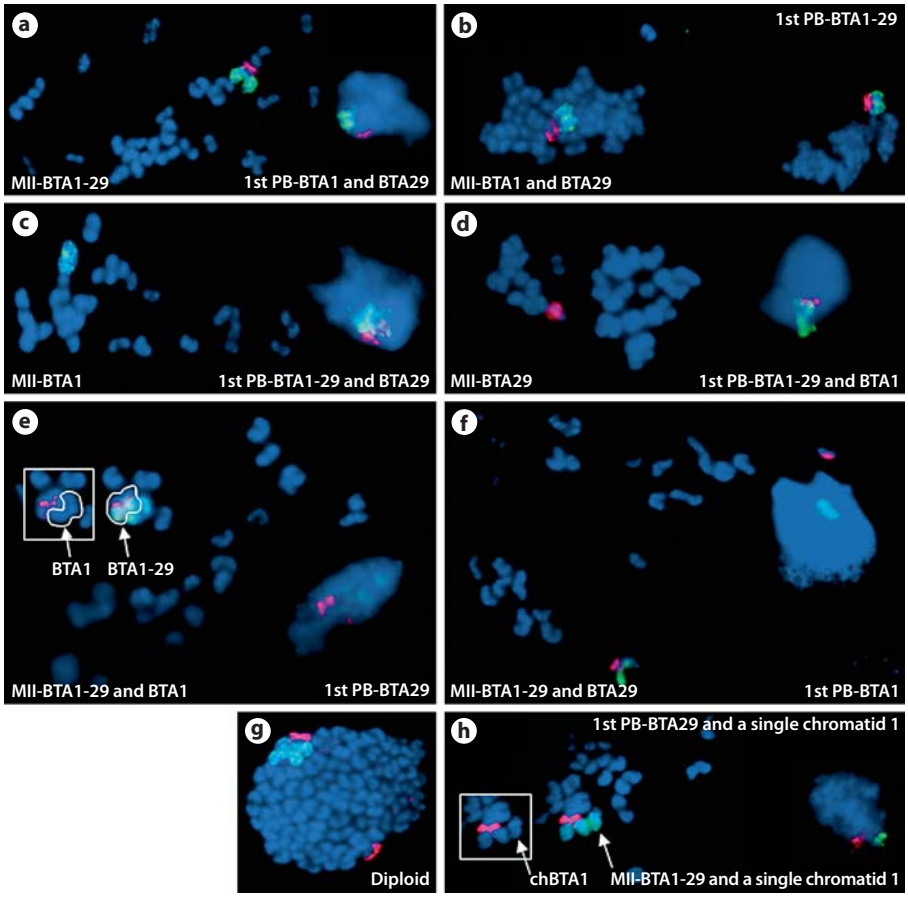


**Fig. 2.** Hybridization of bovine chromosomes BTA1 (green) and BTA29 (red) commercial painting probes on metaphase II oocytes and corresponding first polar body (PB) of t(1;29) heterozygous carrier cows. (**a**) Metaphase II oocyte carrying the translocated chromosome BTA1-29 and its corresponding first PB with free chromosomes 1 and 29; (**b**) metaphase II oocyte with free chromosomes 1 and 29 and its 1st PB with t(1;29); (**c**–**f**) unbalanced metaphase II oocytes and the corresponding 1st PB with (**c**) only chromosome 1, (**d**) only chromosome 29, (**e**) t(1;29) and chromosome 1, and (**f**) t(1;29) and chromosome 29. (**g**) Diploid oocyte with two signals for BTA1 and BTA29 and (**h**) metaphase II with the translocated chromosome BTA1-29 and one chromatid of BTA1 (premature unbalanced separation of sister chromatids) and 1st PB with BTA29. In (**e**) and (**h**) small white squares show a part of the picture counterstained only with DAPI and with chromosome 29 FISH signal for a better understanding of the relative position of the chromosome 1 versus the translocated chromosome. The white arrows indicate the position either of chromosome BTA1-29 or of chromosome 1.

**Table 3.** Distribution of oocytes content for each cow

| Animal | Age | Rate of balanced or normal oocytes[a] | Rate of unbalanced oocytes[a] | Rate of diploid oocytes[b] |
|---|---|---|---|---|
| Cow No. 1 | 6 | 92.5% (111/120) | 7.5% (9/120) | 11.1% (15/135) |
| Cow No. 2 | 11 | 95.8% (69/72) | 4.2% (3/72) | 7.7% (6/78) |
| Cow No. 3 | 10 | 97.9% (48/49) | 2.0% (1/49) | 17.0% (10/59) |
| Cow No. 4 | 6 | 95.5% (64/67) | 4.5% (3/67) | 45.1% (55/122) |
| Total | | 94.8% (292/308) | 5.2% (16/308) | 21.8% (86/394) |

[a] Percentages are calculated without taking the diploid oocytes into account. (Number of conspicuous cells/number of cells analysed).
[b] (Number of conspicuous cells/total number of cells analysed).

proportions of normal/balanced and unbalanced gametes in females were 94.81% and 5.19%, respectively. The latter value is still significantly higher ($P < 0.05$) than that estimated in males (2.76%). Among the four categories of adjacent products, only the percentage of disomy for chromosome 1 was significantly higher (Table 1) for females than for males ($P < 0.001$), the other rates (nullisomies for chromosome 1 or 29 and disomy for chromosome 29) are not statistically different between sexes.

## Discussion

*Equal proportions of normal and translocated-balanced oocytes*

In total or for each cow we did not detect more normal than translocated-balanced oocytes produced by the same alternate segregation mechanism (Table 2). This is quite different from previous findings in humans and mice. In mice, male and female segregation analyses (Gropp and Winking, 1981; Aranha and Martin-DeLeon, 1994) show a distortion in the transmission of the translocated chromosome. Indeed, a predominance of offspring with normal karyotype (higher in males than in females) was demonstrated. In humans, if the Robertsonian translocation is transmitted by male carriers, there is no distortion of the transmission ratio (Pellestor, 1990; Pardo-Manuel de Villena and Sapienza, 2001). Nevertheless, if females are carriers, rather controversial results have been published: Pardo-Manuel de Villena and Sapienza (2001) have found that the translocated chromosome is preferentially transmitted while Munné et al. (2000) have shown that there was an excess of normal oocytes. Our findings argue in favour of a Mendelian inheritance of the translocated chromosome in cow.

*Premature separation of sister chromatids*

We have found three cases of premature unbalanced separation of sister chromatids (PSSC) among the sixteen unbalanced oocytes. These cases represent 18.75% (3/16) of the oocytes that could lead to aneuploid gametes and always involved BTA1 (the chromatid of the free BTA1 was associated with the translocated chromosome, Fig. 2h). This is the first time such a phenomenon is reported in cattle but it has already been described in human (Angell, 1991). Indeed, several cytogenetic studies carried out on unfertilized oocytes or first polar body (Verlinsky et al., 1999; Pellestor et al., 2002; Gutierrez-Mateo et al., 2004; Vialard et al., 2006) demonstrated that PSSC was involved in 60 to 80% of chromosome abnormalities of the oocytes. It is important to point out that in these studies the patients were undergoing assisted reproduction programs often with an advanced maternal age (>35 years) and a normal karyotype. Vialard et al. (2006) suggested that the major cause of chromosome aneuploidy linked to maternal age is PSSC rather than whole chromosome non-disjunction. Moreover, PSSC appears to particularly affect the smallest chromosomes.

Because of the small number of oocytes with PSSC observed, our data have to be taken with caution when compared with human data. In addition, the metaphase II oocytes were not screened for all chromosome abnormalities as only probes for BTA1 and BTA29 were used. However, we always found BTA1 (the longest chromosome in cattle) involved in these PSSC cases and not chromosome 29 (the smallest) which is in opposition with observations made in human. Nonetheless, the PSSC appear not to be related to the age of the donors as examples were found in both older and younger cows.

*Comparison of diploidy rates among cows*

The incidence of diploid oocytes among the four cows was 21.83% on average. If we compare the diploidy rate between cows, three of the four cows show a diploidy rate (from 7.7 to 17%) consistent with that estimated in normal cows (10.7% as described by Yadav et al., 1991 or 11.5% by Lechniak et al., 1996) although cow No. 4 exhibits a high diploidy rate (45.1%). Our data suggested that there is a great inter-individual variability of the diploidy rate.

The comparison of the number of diploid oocytes for cows 1 and 4 (Table 3) suggests that the difference observed (11.1 vs. 45.1%) could neither be explained by a difference of age nor by experimental conditions (Tarin and Pellicer, 1990; Almeida and Bolton, 1995) as they were subjected to exactly the same treatment. We could postulate that cow No. 4 has a failure in the spindle formation (Soewarto et al., 1995) or in the mechanism of extrusion of the first polar body (Lechniak et al., 1996; Pellestor et al., 2002).

*More unbalanced gametes in female than male carriers*

We have found more unbalanced gametes in carrier cows than previously reported for carrier bulls (5.19% vs. 2.76% – corrected rate, Bonnet-Garnier et al., 2006). In Munné et al. (2000) differences between females and males estimated for two Robertsonian translocations (t(13;14) and t(14;21)) were higher (33 vs. 25% and 42 vs. 12%, respectively). However, these results should be considered with caution because of the small number of oocytes analyzed (about 100 for five to twelve patients).

Our study did not show a large variation in the meiotic segregation pattern between sexes. Although the number and the distribution of chiasmata along the chromosomes varies between sexes (Tease et al., 2002; Tease and Hulten, 2004), these parameters did not seem to influence the segregation mode in the case of a Robertsonian translocation contrary to a reciprocal translocation (Tease, 1998; Pinton et al., 2004). Nevertheless, female meiosis produces more unbalanced gametes than male meiosis. This could be explained by the fact that female meiosis is more prone to error than male meiosis probably due to less stringent pachytene and spindle assembly checkpoints (as suggested by Hunt and Hassold, 2002).

In conclusion, to our knowledge this is the first time such a large number of oocytes obtained from females heterozygous for a Robertsonian translocation has been analyzed. The percentage of unbalanced gametes (5.19%, corrected rate) for cows heterozygous for the t(1;29) is smaller than was expected based on human data but is consistent with the decrease of reproductive performances (3–5%) obtained from breeding data. However, it remains two-fold higher than the percentage obtained for spermatozoa (Bonnet-Garnier et al., 2006).

The global impact of the t(1;29) Robertsonian translocation on the fertility of heterozygotes is less than that described for reciprocal translocations (for example in pig: Pinton et al., 2005). Nevertheless, we strongly support the screening program for the t(1;29) Robertsonian translocation for bulls. Indeed, if there is no attempt to control all candidate bulls for being used in artificial insemination (AI) centers, the chromosome abnormality will rapidly spread throughout the population. Thus, if just half of the cows in the French Blonde d'Aquitaine population (i.e. 200,000 individuals) are heterozygous carriers, a 5% decrease in the fertility index will be very important in terms of economical loss. Therefore, we also suggest extending the chromosomal screening to cows and natural mating bulls used for reproduction in herds in order to eradicate this rearrangement more efficiently, since in spite of systematic screening of AI bulls since the 1990s, the estimated frequency of the t(1;29) has remained stable in the French Blonde d'Aquitaine population since 2000.


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