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Centromere Repositioning in Cattle (*Bos taurus*) Chromosome 17

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2. Please provide a Statement of Ethics.

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Cattle · Centromere · FISH

Abstract
Eukaryotic organisms have developed a structure, called centromere, able to preserve the integrity of the genome during cell division. A young bull from the Marchigiana breed, with a normal external phenotype, underwent routine cytogenetic analysis to enter the reproduction center. All metaphases analyzed showed an unusual biarmed chromosome of medium size despite a diploid set of chromosomes (2n = 60,XY). FISH analysis excluded a pericentric inversion or a reciprocal translocation, but highlighted a repositioning of the centromere in BTA17. The satellite DNA was still in an acrocentric position. The telomeres were normally present. The primary constriction on the abnormal chromosome was C-band negative. Finally, the absence of a large genomic deletion in the BTA17 pericentromeric region was demonstrated by both array-CGH analysis and SNP array. To our knowledge, this is the first case of centromere repositioning reported in cattle.

Preservation of genome integrity during cell division is one of the primary objectives for each organism, and, therefore, many mechanisms and structures have been developed to achieve this goal. One of the crucial points is the segregation of sister chromatids during mitotic anaphase, as an error during this step would cause a great imbalance of the genetic content in the daughter cells. For this reason, from early evolutionary stages, eukaryotic organisms have developed a structure able to fully lead this segregation: the centromere. Already in the first cytogenetic studies, this structure has been identified as the region of each chromosome in which the spindle apparatus is attached during chromosome segregation at mitosis and meiosis [Flemming, 1882]. We now know that the centromeric chromatin essentially consists of nucleosomes assembled in the presence of repetitive DNA sequences. In the nucleosome, a variant of the histone protein H3, called CenH3 (or CENP-A), is present and represents the key component of centromeres [Steiner and Henikoff, 2015]. The centromere is the coupling region for a protein complex, called the kinetochore, to which the mitotic spindle attaches [Pesenti et al., 2016]. In human, the repeated DNA sequences in the centromere of each chromosome are composed of tens of thousands of repetitions of alpha-satellite DNA that contains around 400 copies of the CENP-A protein [Bodor et al., 2014].

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The repositioning of the centromere in a chromosome and the subsequent appearance of a neocentromere, already observed in the early 1970s by comparing similar genomes in phylogenetic studies, was explained by the presence of pericentric inversions or other chromosomal rearrangements. In 1979, for the first time, a possible mechanism of centromere transposition was proposed [Dutrillaux, 1979]. Successively, this hypothesis has mainly been confirmed by fluorescence in situ hybridization (FISH) that allows, through the use of appropriate probes, to verify the order of markers present on analogous chromosomes of different species and exclude the presence of pericentric inversions as cause of the repositioning of the centromere [Wienberg et al., 1990]. From studies conducted in humans, we currently know that the formation of neocentromeres is the biological response to avoid the loss of genetic material, for example acentric chromosome fragments, which do not segregate properly during cell division. Acentric DNA fragments are excised to form linear or ring chromosomes. These kinds of neocentromeres are classified as type I and II [Marshall et al., 2008], and they are usually identified in subjects showing clinical problems. Both types result from a rescue process of an acentric chromosome, but type I acentric chromosomes derive from unbalanced rearrangements, whereas those of type II derive from balanced rearrangements.

However, there is a third type of neocentromeres: they are present in a different position than the expected one. They resume the function of the original centromere in the absence of any chromosomal rearrangement. These neocentromeres are very difficult to detect because they do not result in an altered phenotype. Therefore, they are often identified only during a routine screening program as amniocentesis [Warburton et al., 1997]. In the human population, more than 100 cases of type I and II neocentromeres have already been identified [for a review, see Marshall et al., 2008], while only 8 cases of type III neocentromeres have been described [for a review, see Hasson et al., 2011]. What happens to these neocentromeres once they appeared? Since they are not associated with gene imbalances and consequently with clinical phenotypes, they are free to fix themselves in the population and thus become evolutionary new centromeres [Montefalcone et al., 1999]. The presence of these evolutionary new centromeres was observed in many species; it is recognized as an important mechanism of genome evolution [Rocchi et al., 2012].

In cattle (*Bos taurus*), all the autosomes are acrocentric elements. In fact, the centromere is located almost at one extremity of the chromosome, while the sex chromosomes are represented by a large submetacentric X and a small metacentric Y. Thus, the presence of an eventual biarmed chromosome is easily identified, also in metaphases not treated with a banding technique. Despite the thousands of subjects that are cytogenetically analyzed each year, until now all observed biarmed chromosomes were the result of robertsonian translocations [reviewed in De Lorenzi et al., 2008]. Some authors estimate the frequency of these abnormalities at around 0.03% in the cattle population [De Lorenzi et al., 2012]. The displacement of the centromere from the correct position can also result from a pericentric inversion, but so far only 2 cases have been reported, and the X and Y chromosomes were involved [Switonski, 1987; Iannuzzi et al., 2001]. In the present case, we identified an unusual biarmed chromosome of medium size in all metaphases observed despite a correct diploid set of chromosomes (2n = 60,XY). Further analyses excluded a pericentric inversion, while they highlighted a repositioning of the centromere in BTA17. To our knowledge, this is the first case reported in cattle.

Table 1. BACs used in the FISH experiments

BAC	Localization in BTA17, bp (UMD3.1 cattle genome assembly)	Size, kb
200C01	73,170,929–73,298,742	127.3
810C12	532,117–629,425	97.3
395B01	4,665,621–4,730,196	64.6
480C01	6,146,376–6,227,566	81.2
357A10	7,810,266–7,958,744	147.5
712G03	8,104,719–8,221,777	117.1
671G02	8,518,201–8,616,445	98.2

All BACs are from the INRA cattle BAC library. BAC 200C10 maps to the terminal region of BTA17 and was used to verify the involvement of BTA17 in the anomaly.

Materials and Methods

Case Description

A young bull from the Marchigiana breed underwent routine cytogenetic analysis indispensable to enter the reproduction center. At the time of analysis, the bull was 4 months old, and it showed a normal external phenotype corresponding to the Marchigiana breed standard.

Cell Cultures

Peripheral blood lymphocyte cultures were performed following standard methods [Iannuzzi and Di Berardino, 2008] except for the final volume of the cell cultures (5.5 mL). The cultures were incubated for 72 h, and colcemid was added 90 min before cell harvest.

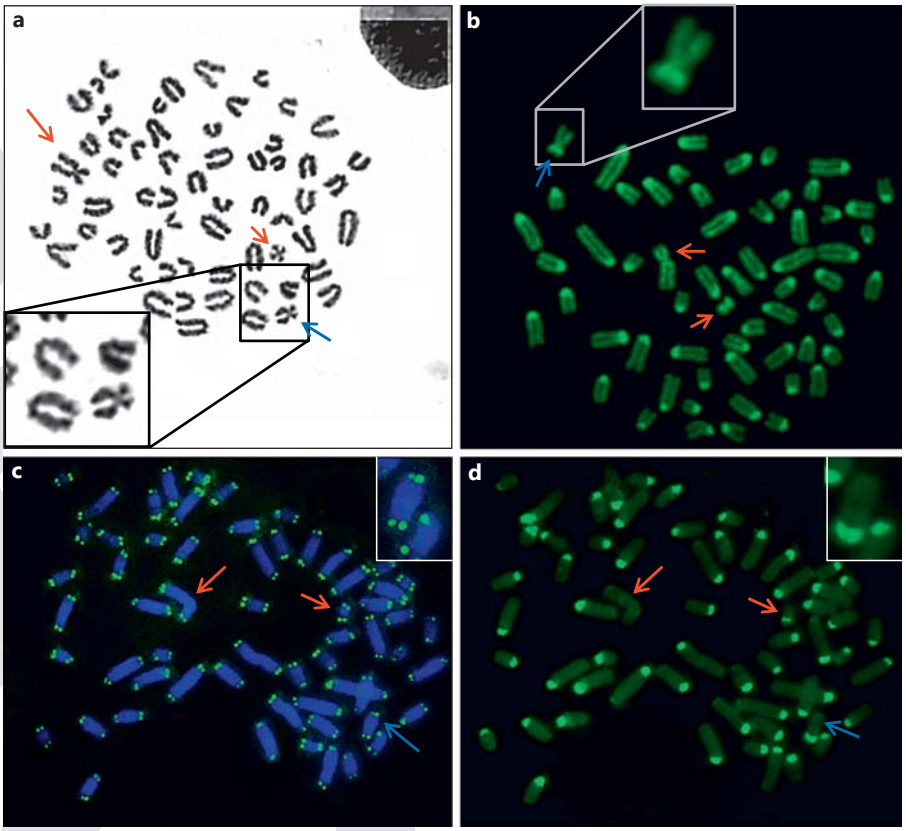

Fig. 1. Characterization of the abnormal BTA17 with the repositioned centromere. **a** Giemsa-stained metaphase. **b** C-banded metaphase. **c**, **d** Metaphase plate after sequential telomere FISH (**c**) and CBA staining (**d**). Telomeric signals are present in all chromosomes including the abnormal BTA17. Sequential CBA staining confirmed the presence of a positive C-band in the telomeric region of the BTA17. Red arrows indicate the sex chromosomes, blue arrows the abnormal BTA17.

FISH Experiments

As probes we used both BACs obtained from the INRA library [Eggen et al., 2001] and bovine satellite DNA previously employed by Chaves et al. [2003]. The localization of these BACs on BTA17 (UMD3.1 genome assembly) is reported in Table 1. DNA for the FISH experiments was extracted according to the method described at the CHORI web site (https://bacpacresources.org/) after overnight growth at 37°C in LB medium supplemented with chloramphenicol. For each FISH experiment, 300 ng of probe DNA was labeled, and FISH was performed as reported in De Lorenzi et al. [2007]. The bovine satellite DNA probes (Sat-I, -III, and -IV) were labeled with biotin or digoxigenin [Chaves et al., 2003], following the hybridization protocols of Iannuzzi and Di Berardino [2008]. Finally, chromosomes were counterstained with Vectashield DAPI H1500 in Vectashield H 1000 antifade solution (Vector Laboratories), and 50 cells were analyzed using Cytovision software.

Sequential Telomere and C-Banding Techniques

Chromosomes were treated with sequential telomere and C-banding techniques. The telomere PNA probe, mapping on all telomeres, was hybridized on metaphase cells using the telomere PNA FISH kit/FITC (Dako Cytomation). At least 50 cells were analyzed using Cytovision software. After the analysis, the coverslip was removed, and the slide was washed in PBST solution, rinsed, and dried before C-banding (CBA). CBA followed the protocols reported in Iannuzzi and Di Berardino [2008], and the same cells as above were analyzed.

Array-CGH Analyses

Array-CGH was performed using the SurePrint G3 Bovine Genome CGH Microarray Kit, 4x180k, with 11.0 kb overall median probe spacing, according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). The positions of oligomeres refer to the Cow Genome October 2007 assembly (bosTau4). Quality of experiments was assessed using Feature Extraction QC Metric v10.1.1 (Agilent), and the derivative log ratio (DLR) spread value was calculated using the Agilent Genomics Workbench software. The experiment has an excellent (0.17) DLR spread value. DLR measures the standard deviation of the probe-to-probe difference of the log ratios and is a measure of array quality: <0.20 is considered as excellent. The bull and ~~his~~ mother were tested, and DNA from a normal male subject was used as control.

SNP Analyses

SNP analyses were performed using GeneSeek Genomic Profiler Bovine 150K (NeoGen) following the producer's instructions. The bull and both parents were tested.

Results and Discussion

All 98 metaphases analyzed showed an abnormal chromosome with a short p arm. Considering that the diploid number was 2n = 60, we first excluded the presence of a

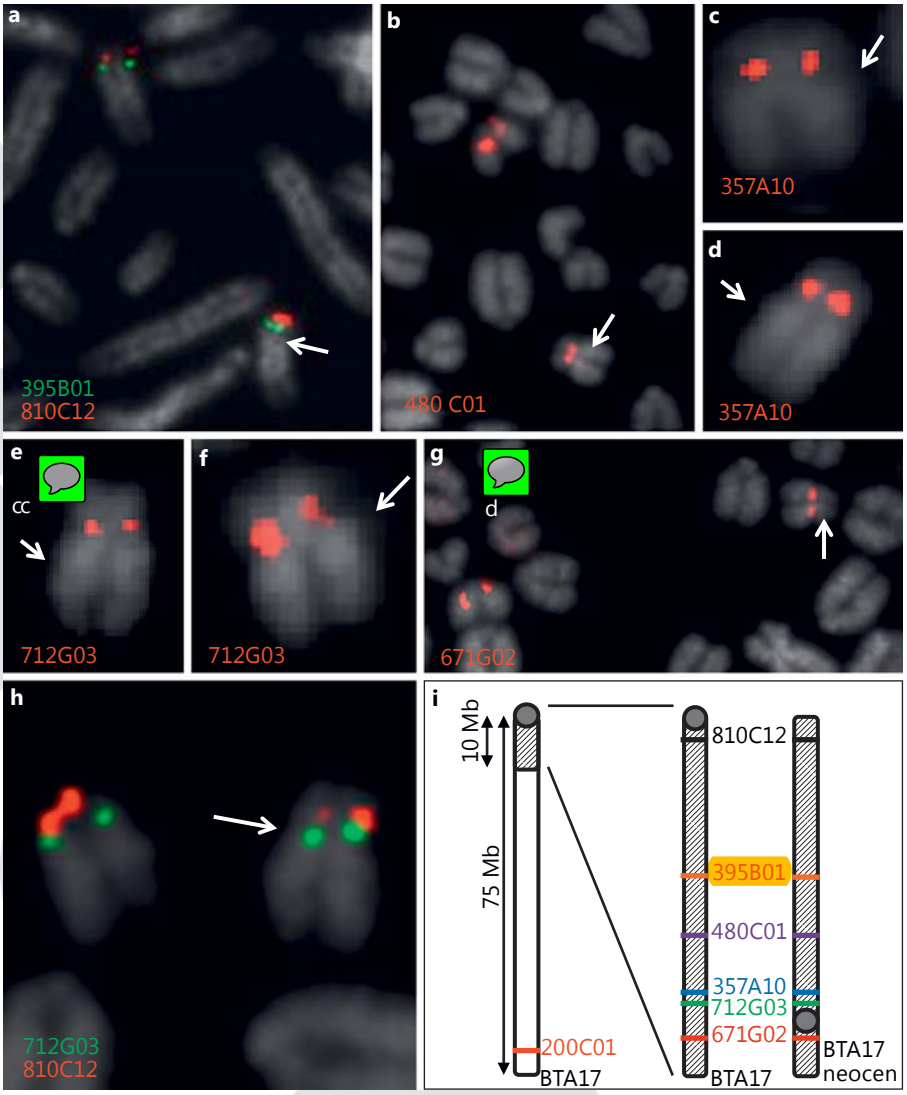

Fig. 2. Characterization of the abnormal BTA17 by FISH with different BAC clones. **a**, **h** Co-hybridization of BACs 810C12 (red signal) and 395B01 (green signal) (**a**) and 712G03 (green signal) (**h**), respectively, in order to determine the order of the BACs on both the normal chromosome 17 and the chromosome 17 with centromere repositioning. **b–f** FISH with BACs 480C01, 357A10, and 712G03 (red signal) proving the positioning of the BACs on the newly formed p arm of the abnormal BTA17. **g** FISH with BAC 671G02 (red signal) showed preservation of the BAC on the q arm of both the normal BTA17 and the BTA17 with centromere repositioning. The neocentromere, therefore, is located between BAC 712G03 and BAC 671G02. The white arrows indicate the abnormal BTA17. **i** Schematic presentation of the position of the BACs on BTA17.

robertsonian translocation (Fig. 1a). In order to ascertain if the abnormal chromosome arose de novo, we analyzed the bull's parents and found that the mother was also a carrier of the abnormal chromosome (not shown). Considering that the centromere appears displaced from an almost terminal to a less distal position, we performed C-banding to highlight this constriction. Surprisingly it revealed that the primary constriction actually present on this abnormal chromosome was C-band negative (Fig. 1b). Finally, ~~considering that~~ the short arm of the chromosome was involved, we studied the presence of telomeric sequences. The telomeres were normally present (Fig. 1c, d).

In order to identify unequivocally the involved chromosome, we preferred a FISH approach rather than conventional banding techniques. Once we identified the chromosome with the neocentromere (using BAC 200C01, not shown), we proceeded with the selection of several specific BACs of BTA17 as previously reported [De Lorenzi et al., 2015]. All BACs tested were on the "new" BTA17p arm (Fig. 2a–f, h) except BAC 671G02 (Fig. 2g) which is located on the newly formed q arm of the abnormal BTA17. Thus, considering the FISH results, we established that the new centromere position is located at around 8.4–8.5 Mb in BTA17 (Fig. 2i; UMD3.1 cattle genome assembly). Moreover, we clearly showed that no inversion was present since the order of the markers was the same in both the abnormal and the normal chromosome 17 (Fig. 2a, h). From these results, we can state that the abnormal BTA17 originated by repositioning of the centromere.

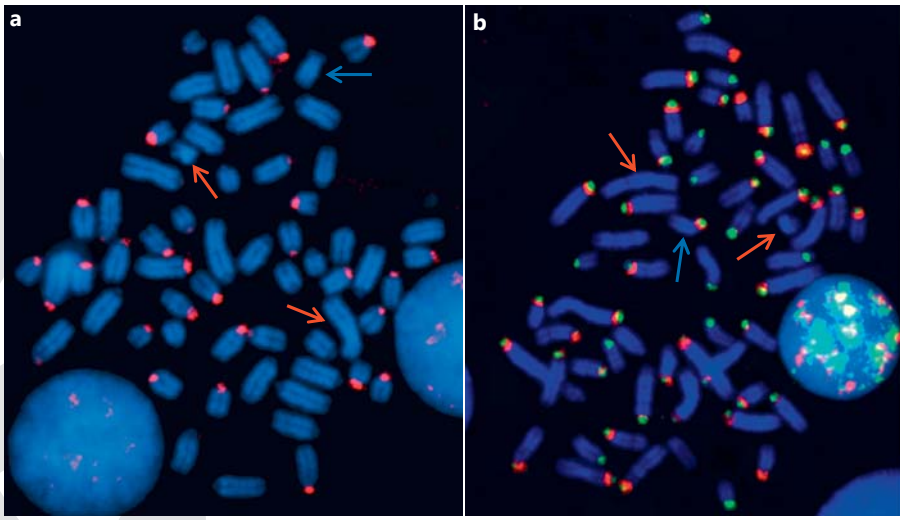

Fig. 3. FISH with satellite DNA probes. **a** Sat-IV (red signal) is not present in BTA17. **b** Dual-color FISH with Sat-I (red) and Sat-III (green) probes. Both Sat-I and -III are present on BTA17. Red arrows show the sex chromosomes, while the blue arrow indicates the abnormal BTA17.

Table 2. Position of the probes in the centromeric portion of BTA17 (first 100,000 bp; Bos_taurus_UMD_3.1.1 genome assembly) and data obtained for the bull and its mother

Probe name	Probe position on BTA17, bp	Analysis	SNP[a]		log2 ratio[b]	
			bull	mother	bull	mother
BovineHD1700000004	5,375	SNP	AA	AC	–	–
BovineHD1700000008	23,517	SNP	TT	TC	–	–
A_63_P12881776	24,129	array	–	–	0.769	0.766
A_63_P12881813	38,078	array	–	–	0.227	0.058
A_63_P12881837	56,032	array	–	–	0.056	0.098
BovineHD1700000025	59,578	SNP	TG	TG	–	–
ARS-USDA-AGIL-chr17-59818-000317	59,818	SNP	GG	TG	–	–
A_63_P12881863	70,304	array	–	–	0.177	0.373
BovineHD1700000028	74,064	SNP	AC	AA	–	–
A_63_P12881889	82,133	array	–	–	0.337	0.103
A_63_P12881912	96,122	array	–	–	0.158	0.026
BTB-01851867	111,319	SNP	AG	GG	–	–
A_63_P12881958	123,944	array	–	–	0.418	0.338

[a] Nucleotides present at the position investigated by SNP.

[b] The normal condition would correspond to a log2 ratio of 0. A log2 ratio intensity of a single-copy loss would be –1, and a single-copy gain would be 0.58. A shift from the normal value of at least 3 consecutive probes is necessary to call an aberration.

Regarding the satellite DNA, normally present in the centromeres of cattle, we performed FISH analyses using probes for Sat-I, Sat-III, and Sat-IV sequences, and the results are shown in Figure 3. BTA17 is characterized by the presence of Sat-I and Sat-III DNA only [Chaves et al., 2000, 2003]. We could clearly show that the neocentromere position does not coincide with the satellite DNA which is still in an acrocentric position. This result also perfectly complies with that reported for neocentromeres in other species, in which the creation of a new centromere does not require the presence of satellite DNA [Burrack and Berman, 2012].

Finally, array-CGH and SNP arrays demonstrated that no loss or gain occurred in the centromeric region of BTA17 or in other BTA17 regions. Depending on the genome position investigated, the log2 ratio intensity certified the absence of a deletion or duplication, whereas for the genome positions investigated by SNP array, the het-

erozygous status of the bull (or the mother, as it carries the same anomaly) certified the absence of a deletion (Table 2). Array-CGH identified several CNVs already reported as polymorphic in previous studies [Bae et al., 2010; Fadista et al., 2010; Liu et al., 2010; Seroussi et al., 2010; Hou et al., 2011; Kijas et al., 2011; Bickhart et al., 2012; Cicconardi et al., 2013].

Moreover, considering that this displacement is not associated with any structural rearrangements, it is also possible that it represents an evolutionary event of creating new centromeres. The appearance of type III neocentromeres is such a rare event that only 8 human cases have been described earlier [Hasson et al., 2011]. The same condition reported here has been observed in several mammals [Cardone et al., 2006], but never before in cattle. In our case, the abnormal BTA17 was inherited from the mother, excluding a de novo event. Considering that the evolutionary new centromeres are not associated with chromosome rearrangements that can jeopardize the viability of cells, they have the possibility to fix themselves and to spread in the population. Thus, assuming no selective effect, the older an anomaly is, the more it is expected to spread in the population. From our 20 years of experience in the laboratory, analyzing more than 2,200 subjects belonging to the Marchigiana breed (as the bull described here), we can assert that no similar case has been reported. It is thus possible that the present neocentromere has a very recent origin and represents the first step of an evolutionary neocentromere path. Since the bull was excluded from reproductive activity, we will not be able to observe the capacity of the abnormal BTA17 to spread in the population nor the eventual phenotypic effect. Since the subject is heterozygous, it would be interesting to observe the behavior of the normal and the mutant BTA17 during meiosis. Up to date, we can only report the data about the reproductive history of the bull's mother as it carries the same anomaly. She required 5 fecundations to generate 2 calves, not a normal condition for the breed considered. As reported for the human cases, the new centromeres are C-band negative, and the DNA is composed of alphoid sequences. Regarding the present case, the C-band is still located in the terminal portion of the chromosome where the centromeric constriction is usually found in the wild-type chromosome.

Therefore, in conclusion, in this report we presented the first case of a centromere repositioning in cattle, and we showed the contemporary absence of large genomic rearrangements.

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Statement of Ethics

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Disclosure Statement

The authors have no conflicts of interest to declare.

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