Sequential Cross-Species Chromosome Painting among River Buffalo, Cattle, Sheep and Goat: A Useful Tool for Chromosome Abnormalities Diagnosis within the Family Bovidae

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Abstract

The main goal of this study was to develop a comparative multi-colour Zoo-FISH on domestic ruminants metaphases using a combination of whole chromosome and sub-chromosomal painting probes obtained from the river buffalo species (Bubalus bubalis, 2n = 50,XY). A total of 13 DNA probes were obtained through chromosome microdissection and DOP-PCR amplification, labelled with two fluorochromes and sequentially hybridized on river buffalo, cattle (Bos taurus, 2n = 60,XY), sheep (Ovis aries, 2n = 54,XY) and goat (Capra hircus, 2n = 60,XY) metaphases. The same set of paintings were then hybridized on bovine secondary oocytes to test their potential use for aneuploidy detection during in vitro maturation. FISH showed excellent specificity on metaphases and interphase nuclei of all the investigated species. Eight pairs of chromosomes were simultaneously identified in buffalo, whereas the same set of probes covered 13 out 30 chromosome pairs in the bovine and goat karyotypes and 40% of the sheep karyotype (11 out of 27 chromosome pairs). This result allowed development of the first comparative M-FISH karyotype within the domestic ruminants. The molecular resolution of complex karyotypes by FISH is particularly useful for the small chromosomes, whose similarity in the banding patterns makes their identification very difficult. The M-FISH karyotype also represents a practical tool for structural and numerical chromosome abnormalities diagnosis. In this regard, the successful hybridization on bovine secondary oocytes confirmed the potential use of this set of probes for the simultaneous identification on the same germ cell of 12 chromosome aneuploidies. This is a fundamental result for monitoring the reproductive health of the domestic animals in relation to management errors and/or environmental hazards.

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Introduction

One of the main goals of cytogeneticists is the characterization of chromosomes by simple, rapid and reliable approaches. In this regard, the classical banding techniques are still the most used procedures since they represent standard and well-established karyotyping methods. This is particularly true for the farm animal populations, whose routine cytogenetic analysis has been performed mainly by the application of classical methods [1].

Despite their wide application, several technical restrictions characterize the classical banding techniques [2] among which the size variations in a chromosomal band or the chromosome itself require a deep knowledge of the banding pattern to resolve complex karyotypes. However, in the last decade, the development of molecular cytogenetic techniques based on fluorescence in situ hybridization (FISH) led to a significant improvement in the accuracy of cytogenetic investigation, representing a valid alternative to the standard methods. In humans, the achievement of 24 colour FISH-based karyotyping (M-FISH, SKY, COBRA [3,4,5]) was the culmination of this technological progress.

Further advancements were reached in humans with chromosome arm-specific [4,6], region-specific [7,8], centromeric [9] and sub-telomeric probe sets [10,11], until arriving to the recent karyomapping [12] which offers a very fine clinical investigation for chromosome imbalances and miscarriage detections.

The applications of FISH techniques in farm animals and humans are very similar and approximately the same level of advancement was reached from animal cytogeneticists in the last two decades. The use of chromosome paintings and DNA probes in domestic animals allowed several important questions to be resolved, including a) detection of chromosome aberrations and complex karyotypes; b) gene mapping and comparative mapping; c) identification of conserved syntenic blocks between species and d) description of chromosome evolution [13,14].
Contrary to humans, the use of multi-colour FISH is still very limited in animal cytogenetics. This is mainly due to the lack of existing commercial probes which are essentially limited to sex chromosomes for most of the domestic species and to two autosomes in cattle for the detection of rob (1; 29) translocation. In addition, only some laboratories of excellence in the world have probes availability [14], thus limiting the application of the method to few research groups. For instance, Kubickova et al. [15] proposed a tri-colour FISH to resolve rob (14; 20) and rob (16; 20) translocations in cattle, later seven-colour FISH using a specific paint pool was used in camels to identify smaller chromosomes [16], whereas recently a pool of 13 chromosome-specific painting probes were used to develop a sequential multi-colour FISH in river buffalo to quickly identify submetacentric chromosomes and gonomes [17].

The aim of this investigation was to use a combination of whole chromosome and sub-chromosomal painting probes derived from river buffalo (Bubalus bubalis, river type, 2n = 50,XY) in a comparative multi-colour Zoo-FISH study on domestic ruminants such as cattle (Bos taurus L., 2n = 60,XY), sheep (Ovis aries L., 2n = 54,XY) and goats (Capra hircus L., 2n = 60,XY) to develop the first comparative M-FISH karyotype. In addition, we report on the application of this pool of probes on bovine secondary oocytes as potential tool for aneuploidy detection during in vitro maturation.

**Materials and Methods**

**Ethics statements**

Procedures were in accordance with the ethical standards of the national ethics committee on research on animal science of 7th June 2011. All institutional and national guidelines for the care and use of laboratory animals were followed. The protocol was approved by the Committee on the Ethics of Animal experiments of the CNR-ISPAAM (Permit Number: 0000391-18/03/2014).

**Cell cultures**

Peripheral blood cultures from eight clinically healthy adult males (two river buffalo bulls, two cattle bulls, two goat rams and two sheep rams) reared in southern Italy were performed following the method described by Iannuzzi and Di Berardino [1]. Four replicates for each sample were prepared according to the conventional cultures protocol and subjected to 20 min of colcemid (0.05 μg/ml) treatment, followed by centrifugation steps, hypotonic (KCl 75 mM) and fixative methanol/glacial acetic acid (3:1) treatments.

**In vitro maturation of COCs and oocyte fixation**

Ovaries were collected from two slaughtered bovine cows and transported to the laboratory within two hours. Cumulus-oocyte complexes (COCs) were collected through aspiration with 21-gauge needles, washed in TCM-199 (Sigma, USA), and examined on Petri dishes under a stereomicroscope. Only oocytes with

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**Figure 1. Sequential multicolour hybridization with 13 river buffalo DNA probes on normal males metaphase spreads.** Specific signals were clearly detected on: a) river buffalo (Bubalus bubalis, 2n = 50) mitosis used as control; b) cattle (Bos taurus, 2n = 60); c) goat (Capra hircus, 2n = 60) and d) sheep (Ovis aries, 2n = 54) mitosis in Zoo-FISH experiments.

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several compact cumulus cell layers and good morphology were selected for the maturation procedure. Groups of oocytes selected from each donor were transferred into 50-mL droplets of maturation medium consisting of TCM-199 + 10% foetal bovine serum (Gibco), supplemented with 0.5 mg/mL follicle-stimulating hormone (FSH) (Sigma), 5 mg/mL luteinizing hormone (LH) (Sigma), covered with sterile mineral oil (Sigma) and allocated in a humidified atmosphere containing 5% CO₂ in air at 39°C for 24 h.

After 24 h maturation, the COCs were incubated for a few minutes in a 1 mg/mL hyaluronidase solution (Sigma) to remove the cumulus cells, washed in Phosphate Buffered Saline (PBS), and exposed to a hypotonic sodium citrate solution (0.8% w/v) for 3 min, followed by KCl (75 mM) treatment for 3 min. The fixation was carried out using cold methanol/glacial acetic acid (1:1) solution. Oocytes were individually fixed at the center of a pre-cleaned slide, air-dried, and kept at 22°C until analysis.

Chromosome microdissection and painting probes preparations

For the production of probes via chromosome microdissection, the river buffalo fixed lymphocyte suspension was spread onto a pre cleaned 24x60 mm coverslip, air dried and then treated for GTG-banding. According to Pauciullo et al. [17], the probes corresponding to the biarmed pairs (from 1 to 5) were produced by dissecting the centromeric area, to avoid unspecific repetitive amplification of the centromeric regions. The probe corresponding to the X chromosome was produced by dissecting the region Xq21–25, analogous to the Xcen region of the bovine chromosome [18]. The probes corresponding to chromosomes 18 and Y were produced by scraping the entire chromosomes.

Briefly, each micro-needle used for microdissection was broken in a 0.2 ml tube containing a collection buffer made of 5X Sequenase reaction buffer (Affimetrix, USA) and water in a final volume of 3.4 μl. On average 15 copies of the same chromosome were collected in each tube. All tubes underwent to topoisomerase I (10 U/μl) treatment at 37°C for 30 min followed by enzyme inactivation at 95°C per 10 min. Highly processive chromosomal amplification was accomplished by degenerate oligonucleotide primer and sequenase ver. 2.0 DNA polymerase (Affimetrix) through a primary DOP-PCR reaction carried out at 94°C for 1 min, 30°C for 1 min and 72°C for 2 min. The enzyme was diluted according to the manufacture’s guidelines and added during the annealing step at every cycle of the reaction for the first 8 cycles. Further 40 cycles of PCR amplification were performed at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min in a reaction volume of 50 μl made of 1X AmpliTaq buffer, 3.5 mM of MgCl₂, 1 pmol of primer, dNTPs each at 200 μM, 2.5 U of AmpliTaq DNA Polymerase (Applied Biosystem).

Each probe was labelled separately by using a secondary DOP-PCR using 2 μL of products from the first reaction as template. Labelling scheme was performed according to Pauciullo et al. [17], with Spectrum Orange-dUTP and Spectrum Green-dUTP (Abbott, USA).

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**Figure 2.** Round of FISH, corresponding homologous chromosomes in river buffalo, cattle, sheep and goat (from ISCNDB, 2000) and super imposed colour for the 13 chromosome/arm specific painting probes.

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Six sequential rounds of FISH were performed on the same slide. Each round was realized by using two probes simultaneously hybridized on the metaphase plate according to Pauciullo et al. [17], with the exception of the second FISH round in which 3 probes (2p, 2q and 18) were used simultaneously. The labeled probes were mixed and each precipitated in absolute ethanol together with 10 μg salmon sperm DNA and 10 μg calf thymus DNA (both from Sigma). The pellets were vacuum-dried and then resuspended in 15 μl hybridization solution (50% formamide in 2X SSC+10% dextran sulfate) for 1 h at 37°C. The probe solutions were denatured for 10 min at 75°C and pre-hybridized for 60 min at 37°C.

Metaphase preparations were denatured for 3 min in a solution of 70% formamide in 2X SSC (pH 7.0) at 75°C. The slides were hybridized in a moist chamber at 37°C overnight. After hybridization, coverslips were removed by a gentle washing step in 2X SSC. The slides were then washed 3×4 min in washing solution (50% formamide in 2X SSC) at 42°C, followed by 3 additional washing steps for 4 min in 2X SSC at 42°C. Slides were counterstained with DAPI (4,6-diamidino-2-phenylindole) solution (0.24 μg/ml; Sigma) in Antifade (Vector Lab).

The slides were observed at 1000x magnification with a Leica DM5500 fluorescence microscope equipped with DAPI, FITC, Spectrum orange specific filters, the FITC/Spectrum orange double filter, and provided with a Cytovision MB 8 image-analysis system (Leica Microsystems, Wetzlar, Germany). Digital images were captured in gray-scale, whereas false colours were created by the image-analyzing system for a reliable evaluation of the painting probes. Approximately 25–30 metaphases were acquired for each slide.

At the end of each round of FISH, the oil for microscope observation was removed from the coverslips and the slides were washed 2×15 min in PBST in a gently shaking, then air dried and immediately reused in the denaturation step for the next round of FISH.

Results and Discussion

Thirteen chromosome-specific painting probes, generated from river buffalo metaphases via chromosome microdissection and the DOP-PCR procedure were sequentially hybridized on river buffalo, cattle, sheep and goat metaphases in a multi-colour zoo-FISH experiment.

Typically 25–30 metaphase spreads per species were analysed. The DNA probes showed excellent specificity on buffalo mitosis, and the cross hybridization revealed very clearly FISH painting signals in the metaphases and interphase nuclei of all the investigated species (Figure 1). Eight pairs of chromosomes corresponding to the 5 sub-metacentric, two gonosomes and chromosome 18 were simultaneously identified in river buffalo. The same set of probes in the cross-species hybridization experiments covered nearly half of the bovine and goat karyotypes (13 out 30 chromosome pairs), and 40% of the sheep karyotype (11 out of 27 chromosome pairs). These results are summarized in Figure 2. Although this set of probes only partially covers the chromosomal make-up of the investigated species, it allowed the first comparative M-FISH karyotype within the domestic ruminants to be developed (Figure 3).

The chromosomal comparison of different species and the detection of similarities between them is not new. For example, extensive comparative studies have taken place between human and cattle [19], whereas painting probes prepared from flow-sorted chromosomes and made available from the laboratory of Ferguson-Smith have been used in comparative studies in a number of species including human, mouse, pig, cattle, dog, lemurs, Indian and Chinese muntjacs, brown brocket deer, chicken, etc...[13]. Correlation between cytogenetic and gene mapping data is amply shown within the Bovidae (cattle, sheep, goat and buffalo), where similarities in banding patterns are a strong indication of homology at the DNA level [20]. However,
Figure 4. M-FISH carried out on bovine in vitro matured secondary oocytes. Specific fluorescent signals were identified on: a) oocyte at the diakinesis/metaphase I stage of the meiosis; b) oocyte at MII and corresponding PB I. Correct chromosomal segregation can be clearly indicated for 11 autosomes and X chromosome.

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The chromosomal identification by classical cytogenetic methods is not suitable also in the analysis of the meiotic metaphases, whose arrangement is usually evaluated in relation of numerical abnormalities. In this regard, the use of whole chromosome paintings in multicolour experiments became extremely useful in the application of preconception genetic diagnosis procedure for the prediction of chromosomal aneuploidies in human secondary oocytes [32].

With the same rationale, the estimation of aneuploidy in the oocytes of the various domestic species and breeds can be considered as an essential step for improving the in vitro production of embryos destined for the embryo transfer industry, as well as for monitoring future trends of the reproductive health of domestic animals in relation to management errors and/or environmental hazards. In this perspective, the complete set of probes herein produced (with the obvious exception of the Y-probe) was hybridized on 20 bovine secondary oocytes matured in vitro to test their potential use for aneuploidy detection. The interpretation of the results is based on the consideration that the first polar body (PB I) is the mirror image of the secondary oocyte metaphase (MII), therefore the lack of any chromosome in the MII (nullisomy) has its counterpart in the corresponding PB, which therefore results disomic and viceversa.

Specific fluorescent signals were clearly identify for each chromosome in all investigated oocytes. Two out of 20 oocytes showed the presence of bivalents (Figure 4a). Although the pairing of the autosomal bivalents and the sex chromosomes is normal, the occurrence of tetrads and the absence of the corresponding PB I reveals the interruption of the in vitro maturation at the diakinesis/metaphase I stage of the meiosis (Figure 4a).

The remaining 18 oocytes underwent a normal meiotic division. Specific fluorescent signals were visible on both MII and corresponding PB I (Figure 4b), thus evidencing the correct chromosomal segregation and therefore the lack of abnormalities for the investigated oocytes.

The scarce availability of commercially available chromosome-specific probes -in domestic ruminants- is a limiting factor for the investigation of aneuploidy rates by FISH. This is particularly evident in cattle where only few chromosomes were investigated and few studies are available so far [33,34]. In addition, the application of other molecular methods like the comparative genomic hybridization (CGH) is not suitable for the analysis of
bovine aneuploidies. In fact, differently to what observed in pig [33], the acrocentric nature of cattle autosomes hampers the chromosomal identification after CGH hybridization. As a consequence, in this species the identification of specific gains/losses of chromosomal DNA can be detected only by FISH. Although no abnormalities were detected in the investigated oocytes, these data confirm the potential use of the river buffalo probes for aneuploidy detection in germ cells, thus opening further opportunity of investigation for clinical cytogenetic applications also in the other species with difficult karyotype.

Conclusions

A DNA collection made of 13 probes generated by chromosome microdissection and DOP-PCR was sequentially hybridised on river buffalo, cattle, sheep and goat metaphase spreads in cross-species hybridization experiments. Nearly half of the bovine and goat karyotypes (13 out 30 chromosome pairs), and 40% of the sheep karyotype (11 out of 27 chromosome pairs) were covered. This allowed the development -for the first time- a comparative M-FISH karyotype for the domestic bovids, which represents a fundamental step for the future achievement of: a) health screening programs of the breeds (highly productive, endangered, indigenous, etc...) related to these species on a molecular cytogenetic basis; b) rapid identification of simple and complex chromosomal rearrangements; c) cross-species hybridization experiments within the family Bovidae and more generally, for comparative evolutionary studies with species of other families; d) resolution of complex karyotypes with particular regard to the detection of hybrid animals; e) evaluation of the aneuploidy level in germ cells as tool for the monitoring the reproductive health of animals in relation to management errors (hormonal imbalances, nutritional and diet mistakes) and/or environmental hazards (mutagens, mitotic poisons) which are known to damage the mitotic/meiotic machinery of the cell.

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Author Contributions

Conceived and designed the experiments: A. Pauciullo DD LI. Performed the experiments: A. Pauciullo A. Perucatti VG DI. Analyzed the data: A. Pauciullo A. Perucatti AI VG. Contributed reagents/materials/analysis tools: A. Pauciullo GC LI. Contributed to the writing of the manuscript: A. Pauciullo A. Perucatti. Revised the article critically for important intellectual content: GC DD LI.

References

1. Iannuzzi L, Di Berardino D (2008) Tools of the trade: diagnostic and research applied to domestic animal cytogenetics. J Appl Genet 49: 357–366.
2. Claussen U, Michel S, Mühlig P, Westermann M, Grunnut UU, et al. (2002) Densitometric chromosome preparation and implications for the concept of chromosome condensation during mitosis. Cytogenet Genome Res 98: 136–146.
3. Schrock E, du Manoir S, Veldman T, Schoof B, Wieringen J, et al. (1996) Multicolour spectral karyotyping of human chromosomes. Science 273: 494–497.
4. Speicher MR, Goyon Ballard S, Ward DC (1996) Karyotyping human chromosomes by combinatorial multi-flour FISH. Nat Genet 12: 368–373.
5. Hanke H, Wianer J, van Gijlswijk RP, Bezkorevek V, Patenier H, et al. (1999) New strategy for multi-colour fluorescence in situ hybridization: COBRA. Cytogenet Cell Genet 84: 156–160.
6. Karlin R, Aihaldstedt-Soini M, Bitter M, Meltzer P, Trent JM, et al. (2001) Chromosome arm-specific multicolour FISH. Genes Chromosom Cancer 30: 105–109.
7. Müller S, O’Brien PC, Fugger-Smith MA, Wienberg J (1998) Cross-species colour segmenting a novel tool in human karyotype analysis. Cytometry 33: 445–452.
8. Chudoba I, Plesch A, Lorch T, Lemke J, Claussen U, et al. (1999) High resolution multicolour-bandng: a new technique for refined FISH analysis of human chromosomes. Cytogenet Cell Genet 85: 156–160.
9. Henegario O, Bray-Ward P, Bazoreke V, Patenier H, et al. (1999) New strategy for multi-colour fluorescent in situ hybridization: COBRA. Cytogenet Cell Genet 53: 65–79.
10. Van den Wyngaarden J, van Gijlswijk RP, Bezkorevek V, Patenier H, et al. (1999) New strategy for multi-colour fluorescent in situ hybridization: COBRA. Cytogenet Cell Genet 53: 65–79.
11. Kahrn H, Ahlstedt-Soini M, Bitter M, Meltzer P, Trent J, et al. (2001) Chromosome arm-specific multicolour FISH. Genes Chromosom Cancer 30: 105–109.
12. Müller S, O’Brien PC, Fugger-Smith MA, Wienberg J (1998) Cross-species colour segmenting a novel tool in human karyotype analysis. Cytometry 33: 445–452.
13. Chudoba I, Plesch A, Lorch T, Lemke J, Claussen U, et al. (1999) High resolution multicolour-bandng: a new technique for refined FISH analysis of human chromosomes. Cytogenet Cell Genet 85: 156–160.
14. Henegario O, Bray-Ward P, Bazoreke V, Patenier H, et al. (1999) New strategy for multi-colour fluorescent in situ hybridization: COBRA. Cytogenet Cell Genet 53: 65–79.
15. Kahrn H, Ahlstedt-Soini M, Bitter M, Meltzer P, Trent J, et al. (2001) Chromosome arm-specific multicolour FISH. Genes Chromosom Cancer 30: 105–109.
16. Muller S, O’Brien PC, Fugger-Smith MA, Wienberg J (1998) Cross-species colour segmenting a novel tool in human karyotype analysis. Cytometry 33: 445–452.
17. Chudoba I, Plesch A, Lorch T, Lemke J, Claussen U, et al. (1999) High resolution multicolour-bandng: a new technique for refined FISH analysis of human chromosomes. Cytogenet Cell Genet 85: 156–160.
18. Henegario O, Bray-Ward P, Bazoreke V, Patenier H, et al. (1999) New strategy for multi-colour fluorescent in situ hybridization: COBRA. Cytogenet Cell Genet 53: 65–79.
19. Kahrn H, Ahlstedt-Soini M, Bitter M, Meltzer P, Trent J, et al. (2001) Chromosome arm-specific multicolour FISH. Genes Chromosom Cancer 30: 105–109.
20. Muller S, O’Brien PC, Fugger-Smith MA, Wienberg J (1998) Cross-species colour segmenting a novel tool in human karyotype analysis. Cytometry 33: 445–452.
21. Chudoba I, Plesch A, Lorch T, Lemke J, Claussen U, et al. (1999) High resolution multicolour-bandng: a new technique for refined FISH analysis of human chromosomes. Cytogenet Cell Genet 85: 156–160.
33. Nicodemo D, Pauciullo A, Cosenza G, Peretti V, Perucatti A, et al. (2010). Frequency of aneuploidy in in vitro-matured MII oocytes and corresponding first polar bodies in two dairy cattle (Bos taurus) breeds as determined by dual-colour fluorescent in situ hybridization. Theriogenology, 73: 523–529.
34. Pauciullo A, Nicodemo D, Cosenza G, Peretti V, Iannuzzi A, et al. (2012). Similar rate of chromosomal aberrant secondary oocytes in two indigenous cattle (Bos taurus) breeds as determined by dual-colour FISH. Theriogenology 77: 675–683.
35. Hornak M, Jeseta M, Musilova P, Pavlok A, Kubelka M, et al. (2011) Frequency of aneuploidy related to age in porcine oocytes. PLoSOne 6:e18892.