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Review Paper

Chromosomal Anomalies and Infertility in Farm Animals: A Review

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ABSTRACT

Veterinary cytogenetics is an area of genetics that deals with normal or abnormal chromosomes of animals. Chromosome abnormalities in cattle can cause significant adverse effects on fertility through failure of production of viable gametes or early embryonic death that consequently leads to great economic loss. Chromosomal aberrations can occur as numerical errors or structural rearrangements usually without causing phenotypic abnormalities on carrier animals. According to current knowledge on chromosomal abnormalities, Robertsonian translocation (ROB) that involves chromosome 1 and 29 represents the most common form of aberration found in cattle of various breeds. Other less commonly encountered abnormalities in cattle include reciprocal translocation, chimerism (including freemartins), mosaic and rarely sex chromosome aberrations. A similar trend in incidence of abnormalities has been observed in sheep and goats although systematic studies are limited. Centric fusion that involves different chromosomes but not specific to chromosome (1;29), is the most common abnormality, followed by chimerism, sex chromosome abnormalities, and rarely deletions and inversions. In swine, reciprocal translocations are the most common abnormalities with significant economic loss due to reduction in litter size by up to 50%. This is followed by chimerism for sex chromosomes. Unlike cattle, incidence of ROB (1,29) in pigs is very rare. In domestic buffaloes, sex chromosome abnormalities are the most common found associated with infertility due to extensive damage to sex adducts. Freemartinism is the most prevalent form of all sex chromosome abnormalities detected in buffalo. However, translocations are rare incidences in buffalo. Sound breeding programmes and successful cattle production depend on minimizing

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and preventing all kinds of possible causes of reproductive failure in both females and males. Unlike many other causes of reproductive failure, little attention appears to be given towards cytogenetic anomalies examination at the time of breed selection for genetic improvement, as well as during investigation of causes of reproductive failure in farm animals. This is especially true in the tropical regions where cytogenetic studies are limited and their significance is poorly understood. This review provides an overview and update on chromosomal anomalies, their effect on fertility of farm animals and screening methods, with subsequent aim of drawing the attention of concerned bodies to make an effort towards understanding the magnitude and significance of the problem in the tropics by applying feasible and available biotechnological tools pertinent to cytogenetics. This consequently would help to design possible strategies to prevent and control the propagation of chromosomal aberrations in farm animals in the tropics.

Keywords: Chromosomal anomalies, cytogenetics, farm animals, reproductive failure, screening techniques

INTRODUCTION

Maintaining high fertility rate and economic return is the main goal of animal farming enterprises. Fertility is a multifactorial phenomenon which is influenced by several factors that often overlap including nutrition, disease, hormonal disturbance, reproductive functions, sperm quality, physical soundness, environment, management and genetics. Cattle breeders are well aware of the effect of most of these factors and take measures to minimise associated problems at the time of animal selection. However, it is very rare to see breeders much worried about genetic abnormalities related to chromosomes especially in areas where cytogenetic studies are limited such as in the tropics including South East Asia and Africa. The magnitude and significance of occurrence of chromosome anomalies are also little understood. Although reduced fertility occurs mostly due to a combination of several factors, in some instances, it can be traced to a single genetic factor. For example, with regard to chromosome anomalies, bulls with Y-autosome reciprocal translocations (Iannuzzi *et al*., 2001) show an apparent normal phenotype but found with oligozoospermia or azoospermia. Thus, it is always important to consider cytogenetic anomalies as possible causes of reproductive failure during investigation and keep updated on their occurrence, distribution, significance and related techniques for investigation.

CYTOGENETIC ANOMALIES AND THEIR EFFECT ON FERTILITY OF FARM ANIMALS

Cattle

Domestic cattle of *Bos taurus*, *Bos indicus* and their crosses normally possess a diploid number of 60 chromosomes, which comprises 29 pairs of autosomes and a pair of sex chromosomes (XX in females and XY in males). Structurally, all the 29

pairs of autosomes and the X chromosome are acrocentric and sub-metacentric, respectively, in both *Bos taurus* and *Bos indicus* breeds. The only difference is in the Y chromosome, which is sub-metacentric in *Bos taurus* but acrocentric in *Bos indicus* breeds (Lightner, 2008).

Chromosomal abnormalities can occur as a result of alterations in number such as polyploidy (the condition of having the entire set of chromosomes beyond the basic set with an exact multiple of the haploid number) and aneuploidy (the condition of having or missing one or more chromosomes from the basic set), or as a result of structural changes which consist of translocations, deletion, duplication and inversion. Such chromosomal abnormalities which involve the chromosome number and structure including their frequency and phenotypic effects are dealt under clinical cytogenetics (Popescu, 1996). The initiation of conducting clinical cytogenetic investigations is usually associated with observation of fertility problems, congenital defects or as a part of an eradication programme for chromosome aberrations (Iannuzzi *et al.*, 1993; Lioi *et al*., 1995; Popescu, 1996; Molteni *et al*., 1998; 2007; Ducos *et al*., 2000). Variable degrees of infertility in cattle of different breeds, which are carriers of numerical or structural chromosomal abnormalities, have been reported in previous studies (Muñoz *et al*., 1994; Rubes *et al*., 1996; Schmutz *et al*., 1997; Molteni *et al*., 1998; Tanaka *et al*., 2000). Anomalies such as translocations (Gustavsson, 1979; Popescu, 1996; Ducos *et al*., 2000, 2007, 2008), tandem fusion and chimerism for sex cells (Pinheiro *et al*., 1995), sex chromosome aneuploidy (Gustavsson, 1977; Citek *et al*., 2009) are among the important abnormalities in cattle reported to be associated with variable degrees of infertility. Other abnormalities such as deletions and insertions are not commonly found in animals as they are lethal at early stage of an embryonic life.

As it is well recorded, the most frequently reported and widely distributed chromosomal aberration in cattle of various breeds is centric fusion, also known as Robertsonian translocation (ROB). Robertsonian translocation involves the fusion of two acrocentric chromosomes at their centromeres thereby forming a single large sized meta/sub-metacentric chromosome and reducing the total chromosome number by one in heterozygous carriers or by two in homozygous cases. The most prevalent form of ROB is the fusion between the 1st and the 29th chromosomes, ROB (1;29) (Gustavsson, 1979; Schifferli *et al*., 2003; Ducos *et al.*, 2008; De Lorenzi *et al.*, 2012). It was first identified in Swedish Red and White cattle (Gustavsson & Rockborn, 1964). Since then, several other reports have described this translocation in scores of different cattle breeds from all six continents with a considerable variation in incidence among breeds (Popescu & Pech, 1991; Lightner, 2008; Ducos *et al.*, 2008). Both male and female cattle with ROB (1;29) are carriers and appear phenotypically normal (Iannuzzi *et al*., 1993; Rubes *et al*., 1996). However, these animals have variable degrees of infertility because of production

of unbalanced or aneuploid gametes that result in the formation of zygotes with unbalanced chromosomal constituents that subsequently results in early embryonic death (Blazak & Eldridge, 1977; Wilson, 1990; Schmutz *et al*., 1991; Tateno & Miyake, 1994; Popescu, 1996; Molteni *et al*., 2005). In cattle carrying ROB (1;29), the observed value of unbalanced gametes is 2.76% in sperms and 4.06% in oocytes (Bonnet-Garnier *et al*., 2008).

Since Gustavsson (1979) has demonstrated the effect of ROB (1;29) in reducing fertility in heterozygous carrier cattle, several other researchers have also confirmed its adverse effect on fertility based on key reproductive performance indicators at different times (Schmutz *et al*., 1991; Popescu, 1996; Molteni *et al*., 2005; Ducos *et al*., 2008). For instance, Schumtz (1990) stated that bulls carrying ROB (1;29) cause abnormal embryos in about 10% of conceptions, while cows carrying this ROB cause 20% of embryos to be abnormal. A diminished non-return rate of 5 and 7% at 28 and 56 days post insemination, respectively (suggesting embryonic mortality), was reported from females sired by bulls heterozygous for ROB (1;29) (Dyrendahl & Gustavsson, 1979). Similarly daughters of carrier heterozygous bulls have been reported to have reduced fertility and greater culling rate than daughters of normal bulls (Schifferli *et al*., 2003). A 5 – 10% reduction in fertility by increasing the embryonic death has been reported by Popescu (1996). Another study, which compared heterozygous carrier cows for ROB (1;29) with cows having normal karyotype, showed that carrier cows required higher insemination index and resulted in lower fertility characterised by lower percentage of pregnancy following first insemination (Kovacs *et al*., 1992). Moreover, carrier cows were also reported to show delayed age at first breeding and calving, prolonged calving interval, decreased calving rate and prolonged number of days open compared to the normal cows. Although not as frequent as ROB (1;29), several other ROBs were also identified, in which all 29 pairs of chromosomes had been involved in at least one ROB described in the literature (Lightner, 2008). The reduction in fertility in cows was observed not only for ROB (1;29) but also for other forms of ROB translocation such as ROB (26;19) (Ducos *et al.*, 2008). Based on experimental studies, heterozygous cows for ROB (26;19) sired by carrier bull compared with normal cows sired by the same carrier bull showed an increase in percentage of negative service, irregular return to heat as well as increase in average calving interval (Ducos *et al.*, 2008). Heterozygosity for a ROB theoretically leads to formation of normal or balanced gametes and through meiotic nondisjunction to the formation of aneuploid gametes. Participation of such aneuploid gametes in fertilization results in monosomic zygotes (a form of aneuploidy which occurs when one chromosome is absent from the normal diploid number) and trisomic zygotes (a form of aneuploidy which occurs when there are three copies of a chromosome instead of the normal diploid number) which die

during gestation (Blazak & Eldridge, 1977). The magnitude of reproductive disturbances depends on the frequency with which non-disjunction occurs at meiosis, viability of aneuploid gametes and zygotes.

Even though it has not been described as often as ROB, there are some reports on reciprocal translocation (RCT) in cattle which are phenotypically normal but with poor fertility (Ducos *et al*., 2000; Iannuzzi *et al*., 2000) and even with infertility/ azoospermia problems (Molteni *et al*., 2007). Although ROB (1;29) represents the most prevalent abnormality in cattle, there is a debate about the true incidence of RCT. De Lorenzi *et al.* (2012), who investigated real estimate of reciprocal translocations based on 15 years of data on cytogenetic results using mathematical and simulation approach, concluded that only 16% of RCT could be detected using simple Giemsa technique. It was shown that RCT could be present in no less than 0.14% of cattle subjects, a frequency five times higher than the de novo ROBs (that involves chromosomes other than chromosomes 1 and 29) (De Lorenzi *et al.*, 2012). According to Ducos *et al.* (2008), based on a screening programme from Italy, they reported an incidence of 7.1% for ROB and 0.03% of RCT. Out of the 7.1% of ROB incidences, majority were the endemic form (1;29), while the remaining 0.03% of the incidences were due to de novo ROB translocations, a frequency very similar to RCT. This implies the need to employ more efficient cytogenetic techniques such as chromosome banding and molecular techniques in order to avoid failure of detection and subsequent underestimation of the incidence of RCT in cattle. Other less commonly encountered anomalies compared to ROB in cattle include chimerism for sex chromosomes including freemartins and rarely sex chromosome aneuploidy (De Lorenzi *et al.*, 2012). Freemartinism is considered as the common form of sex chromosome anomalies in cattle. In 86.4% of females born co-twin together with males globally presented an XX/XY blood chimerism, while the remaining 13.6% had normal XX karyotype (De Lorenzi *et al.*, 2012).

Most of these chromosomal abnormalities reported so far are mainly from European breeds of cattle. Currently, only the incidence of ROB (1;29) in many breeds of cattle populations in Europe is known. It has been reported as high as 65% incidence in Barrosas breed of Portugal (Figueiredo & Iannuzzi, 1993). Following reports of the incidence of ROB (1;29) and its deleterious effect on fertility in 1979, a control programme was established in many countries of Europe whereby animals intended for breeding are cytogenetically evaluated and selected against chromosomal abnormalities (Popescu, 1996). Investigation and monitoring activities were increased in many countries of the world in order to bring ROB (1;29) under control. Eradication programme of ROB (1;29) carrier bulls in Sweden was introduced in 1969 to eliminate the translocation and has subsequently resulted in increased fertility of the whole population (Gustavsson, 1979). In Australia and Great Britain, cytogenetic evaluation

is a requirement for all cattle imported. In Europe, generally speaking, it has been a requirement for imported breeding animals or semen is free of chromosomal abnormalities.

In contrast, despite the huge cattle populations that exist in the tropics including Asia and Africa, and the poor reproductive performance prevailing, studies on chromosomal abnormalities including ROBs are very limited. There is one published report on incidence of a case of ROB (2,28) in Vietnamise cattle (Tanaka *et al*., 2000). Reports of cytogenetic anomalies including ROBs from Africa with large cattle population are also very few (Pathiraja *et al*., 1985; Nel *et al*., 1985) with no later research investigations available. These studies demonstrated incidence of ROB (1;29) in one of 10 Rahaja bulls of Nigeria and 10.2 % of Nguni indigenous cattle breeds of South Africa, respectively. Although such reports were alarming type for the possible high occurrence in the region, further investigations appeared to be discontinued. As a result, the frequency and significance of chromosomal anomalies remain largely unknown. It is expected that the incidence of chromosomal anomalies might have been also worsened by genetic inheritance and introduction of foreign breeds of cattle/germ plasm into these regions in the absence of a control system for cytogenetic anomalies.

Sheep and Goats

Studies on chromosomal anomalies and screening programmes in small ruminants were not as systematic as they were for cattle. Considering the close similarity in the structure of chromosomes of cattle with goats and sheep, the pattern of incidence in the type of chromosome anomalies might be similar. However, this needs a wider investigation for validation.

Centric fusion is the predominant abnormality in sheep and goats though there is some degrees of variation in terms of breed and number of animals evaluated (Long, 1990; Goncalves *et al*., 1992; Dai *et al*., 1994; Switonski & Stranziger, 1998), with most of the studies limited to less than 100 animals. Among the commonly detected forms of centric fusion including ROBs involve chromosome numbers 5,26; 8,11; 7, 25 in sheep (Dai *et al*., 1994) and 5,15; 6;17, 2,12; 6,15; 10,12; 3,17 in goats, mostly of Saanen breeds and reported mainly from Europe, Israel and Brazil (Goncalves *et al.*, 1992; Switonski & Stranziger, 1998). Unlike cattle, incidence of ROB translocation that involve chromosomes 1;29 in sheep and goat is rare; one report in sheep that belong to the Portuguese Churra Terra Quente breed (Chaves *et al*., 2003). In a cytogenetic study that involved 205 goats related to a carrier male goat to a ROB (5;15), the prevalence of heterozygous carriers found was 29.27%, while 4.88% of them were homozygous carriers for the translocation (Goncalves *et al.,* 1992).

Data on the effects of individual chromosome abnormalities on fertility of sheep and goats are rare. It was reported in sheep that despite several ROB translocations described, reduction in fertility was not

observed (Long, 1997). Nevertheless, presence of association between numerical chromosome abnormalities and reduction in fertility in goats has been shown by Bhatia and Shanker (1996). In this study, goats that showed late maturity, anestrus, repeat breeding problem and not conceiving despite regular estrus were investigated for their chromosomal status and compared with their normally performing counter partners. The results showed that 51% of those animals with reproductive problems exhibited various forms of numerical chromosomal abnormalities such as mosaic for polyploidy (17 diploid/tetraploid mosaic; 2 diploid/triploid/tetraploid mosaic, 3 monosomy/diploid mosaic and 1 chimeric) but none of the control animals exhibited such abnormalities. A higher frequency of leucocyte chimerism for sex chromosomes (XX/XY) was also detected in sheep breeds from Poland (De Lorenzi *et al*., 2012). However, the frequency of freemartinism (XX/XY chimerism) was known to be low in sheep compared to the incidence in cattle and buffalo, despite the common co-twin birth in sheep (Brace *et al*., 2008; Di Meo *et al*., 2010). Other less commonly reported chromosomal abnormalities include sex chromosome abnormalities, deletions and inversions (De Lorenzi *et al*., 2012). As cytogenetic studies in sheep and goat are yet limited, making generalizations as to the magnitude of the various types of abnormalities, their significance and influence on fertility is difficult. Therefore, more systematic cytogenetic investigations involving larger populations should be encouraged for a better conclusion.

Buffaloes

The domestic buffalo (*Bubalus bubalis*) are also among the important farm animals that play major roles in the animal production sector especially in the Asian continent. In India, for example, dairy buffaloes contribute 60% of the total milk produced by the dairy industry (Chauhan *et al.,* 2009); hence, they are of great economic importance. The domestic buffalo have been classified into two subspecies. The first type is the river buffalo raised in most areas from India to Egypt and some South and East Europe. The second type is the swamp buffalo of the South East Asia (Bongso & Hilmi, 1982; Iannuzzi, 2007; Supanuam *et al.,* 2009). The chromosome complement of the two types of buffalo and their crossbreed was described using Giemsa and centromeric banding techniques by Bongso and Hilmi (1982). The diploid chromosome number of the River buffalo (Murrah) is 50, while that of the Swamp type (Malaysian Kerbau) is 48. The F_1 hybrid between the two buffalo types has 49 chromosomes. It was also described that the largest two metacentric chromosomes of the Swamp buffalo is the result of a tandem fusion between the telomere of the short arms of chromosome 4 and the centromere of chromosome 9 of the River buffalo (Bongso & Hilmi, 1982; Hishunuma *et al.,* 1992).

When standard nomenclature for chromosomes of most domestic species of animals was established in the 1980s and later revised in the 1990s, the swamp and the river buffaloes were not considered

among the species. Hence, cytogenetic studies during that time were focused on understanding the normal chromosome structure and description of the region of the tandem fusion in chromosome number one of the swamp type towards the establishment of standard karyotype (Hishunuma *et al*., 1992) but with little attention given to clinical cytogenetics. Clinical cytogenetics for investigation of chromosomal anomalies in buffaloes was developed much later compared to other domestic animals (Iannuzzi *et al*., 2005; Iannuzzi, 2007; Di Meo *et al*., 2008; Chauhan *et al.*, 2009). Unlike cattle, systematic cytogenetic screening programmes for chromosome anomalies in both river and swamp buffaloes are rare and most of the reports are individual cases or from a group of animals selected for reproductive problems. The most common chromosome abnormality reported in buffalo (mostly from river type) is sex chromosome abnormality which includes X-trisomy (Parakash *et al.,* 1994; Iannuzzi *et al*., 2004), X-monosomy (Iannuzzi, 2000), Sex-reversal syndrome (Iannuzzi *et al.*, 2001; 2004), XXY-syndrome (Patel *et al*., 2006) and XX/XY mosaicism (freemartinism) (Iannuzzi *et al.*, 2005). Nevertheless, unlike cattle, autosomal chromosome abnormalities such as translocations have rarely been reported in river buffalo.

Almost all the sex chromosome abnormalities reported in river buffalo were associated with sterility due to damage to the internal sex adducts of the females (Iannuzzi, 2007) and inability to produce sperm (azoospermia) in males (Chauhan *et al*., 2009). Freemartinism (XX/XY chimerism) is the most common sex chromosome abnormality in river buffalo as in cattle. For example, in a study that involved 134 river buffaloes with reproductive problems after maturity (13 males, 2 male co-twins and 119 females which had failed to become pregnant in the presence of bulls), 25 animals (20.7%) were found to carry sex chromosome abnormalities. Of which, majority (20, 18 females and 2 males) of them were XX/XY free-martins and all the female carriers were sterile (Di Meo *et al.*, 2008). In another study by Iannuzzi *et al*. (2005) from Italy, it was also shown that freemartinism as the major sex chromosome error found in river buffaloes with reproductive problems. Of the 42 animals studied, 10 freemartins (8 females and 2 males) were found. Six of the eight females showed normal body conformation, vagina and clitoris, while two showed some male traits (tight pelvis). The two males were apparently normal with only a reduced size of one testicle in one animal. Based on clinical observations performed in the internal reproductive organs of the female carriers, serious damages varying from complete lack of internal sex adducts (closed vagina) to hypoplasia of Müllerian ducts and absence (or atrophy) of ovaries were revealed (Iannuzzi *et al.*, 2005). According to histological examination of Murrah and Swamp buffalo hybrids, it has been also revealed that a large proportion of degenerating spermatocytes and abnormal spermatids in the process of spermatogenesis

leading to unbalanced gametes. These unbalanced meiotic products may probably lead to selection against such spermatozoa or early embryos after fertilization causing subnormal fertility (Bongso *et al.*, 1983).

Almost all examined XX/XY freemartin cases in buffalos were from single births, which means that the male co-twin dies during the early embryonic life and a single female birth arises (Iannuzzi, 2007). This makes it difficult to suspect the single born female of being a freemartin and will be allowed to stay in the farm until sexual maturity and show reproductive problems causing great economic loss. This indicates the importance of extending studies to all females (born single or co-twinned) with reproductive problems. Moreover, to minimize the economic loss due to raising a sterile female by waiting until it reaches the age of maturity and manifests reproductive problems, early clinical examination and detection aided by male traits expressed by many of carrier females and subsequent cytogenetic evaluation should be encouraged for prompt elimination from the farm.

Pigs

The pig is the most extensively evaluated animal for which accurate estimate of the prevalence of structural chromosomal anomalies is available (De Lorenzi *et al.*, 2012). An estimated 0.47% prevalence of balanced chromosomal rearrangements was recorded from a large number of young boars karyotyped (Ducos *et al*., 2007). In pigs, where chromosome banding techniques used to be applied as screening method, RCT is the most frequent form of chromosomal anomaly encountered. There are 33 different RCTs known worldwide (mostly reported from Europe), which are responsible for significant economic loss due to a reduction in litter size by 30 - 50% (Ruth *et al*., 1993; Rodriguez *et al*., 2010). Early embryonic mortality accounted for the reduction in litter size (Popescu *et al*., 1984). Chimerism for sex chromosomes is the second most commonly reported abnormality in pigs associated with hypoprolificity and intersexuality, while ROBs were rarely detected (Ducos *et al.,* 2004; Ducos *et al.*, 2008) unlike cattle. In human newborns, where more advanced screening methods used to be involved, the frequency of ROB and RTC are reported to be very similar (Van Assche *et al.*, 1996) and their frequencies were higher in couples experiencing repeated pregnancy losses (De Braekeleer & Dao, 1991).

Although the impact of chromosomal translocations on fertility is widely acknowledged, there exists some degree of differences in terms of species and type of translocation. In pigs, according to several studies, the proportion of unbalanced sperm ranged between 47.83 and 24.33% depending on RCT type (Pinton *et al*., 2004). The proportion of chromosomal unbalanced sperm produced by translocation carriers in humans ranged from 19% to more than 80%, which appeared to be again dependent on the type of translocation (Benet *et al*., 2005). According to Ferguson *et al*. (2008), it has been shown that RCT can induce failure in synaptonemal complex and

arrest of meiosis process. These imply that the impact on fertility posed by RCP is much higher than ROB, possibly making it worthy of involving more advanced techniques for future investigation to understand its actual incidence in cattle.

Apart from the difference in the cytogenetic technique employed so far for screening, the relative abundance of ROB than RCT in cattle compared to other species of animals including swine and humans might be attributed to the difference in the structure of chromosomes. In cattle, all the 29 chromosome pairs are acrocentric in morphology with one arm only and consequently centric fusion (ROB) could be more favourable to occur than in pigs and humans where one armed chromosomes make only a small part of the whole chromosomes karyotype (De Lorenzi *et al.*, 2012).

CYTOGENETIC TECHNIQUES FOR SCREENING CHROMOSOMAL ABNORMALITIES IN ANIMALS

Blood Cell Culture

Blood cell culture involves stimulating lymphocytes to grow by various lectins used as mitogens (Pokeweed, PHA, Concavalin A). Multiplying cells are stopped at metaphase using colcemid. The treatment of cells by hypotonic solutions enables to obtain well dispersed metaphase chromosomes for analysis using conventional Giemsa staining or banding techniques based on which karyotypes are constructed.

Conventional Method of Blood Cell Culture

Blood collected aseptically is the sample of choice for culture in domestic animals for screening chromosomal anomalies. About 1 mL of whole blood or buffy-coat layer separated by centrifuge can be used to initiate the culture. The culture media commonly consists of RPMI1640 (8 mL) foetal calf serum (2 mL), mitogen (e.g., Pokeweed mitogen, 0.1 mL), Antibiotics (0.1 mL Penicillin-Streptomycin) and L-glutamine (50 µL). In the case of whole blood culture, sodium heparin (50 µL) is also added to prevent coagulation. The cell culture is incubated for 72 hours at 37.5°C by agitating it once a day. Towards the end of the culture, colcemid (20 – 50 µL) is added for a period of 30 – 60 min to stop the cell cycle. The optimum concentration of colcemid and treatment plays an important role and hence, needs to be checked. To our experience, for bovine sample, a concentration of 40 µL for a treatment period of 30 min resulted suitable and elongated chromosomes. The culture is finally treated with a hypotonic solution (warm 0.075 M of KCL) for 20 min. After centrifuging the culture and removing the supernatant, the cells are fixed using Carnoy's fixative (3 part methanol, 1 part glacial acetic acid) added drop by drop. After exchanging the fixative three times by centrifuging and discarding the supernatant, the cell culture is left overnight at 4°C before making drops on slide and staining.

Early 5-bromo-2-deoxyuridine (BrdU) Incorporation in Conventional Cell Culture:

This involves addition of BrdU (15 µg/mL) to the cell cultures 8 h before harvesting. After 2.5 h, centrifuge the culture and remove the supernatant. The culture is restarted with complete fresh medium containing thymidine (10 µg/mL) for further 5.5 h. Ethidium bromide (5 µg/mL) 2 h before harvesting and 20 µL of colcemid 30 - 60 min before termination is added. For harvesting, the same protocol described for conventional blood culture above is followed. This technique provides good amount of 'pro-metaphase' plates suitable for G-banding and C-banding and is reported to offer better results in animals such as pig (Iannuzzi & De Berardino, 2008).

Synchronized Blood Culture

Thymidine is one of the commonly used chemical for culture synchronisation. For this purpose, a 200 µL thymidine (300 µg/mL of final concentration) is added to the 10 mL culture after 48 h of incubation. After 17 h of incubation with thymidine, the cell suspension is centrifuged and the supernatant is discarded. The cells are then washed with either fresh medium or with Hank's Balanced Salt Solution (HBSS), centrifuged at 1800 rpm for 8 min followed by removal of the supernatant. The culture is continued in a complete fresh medium that contains all the components, except the mitogen, for further 5.5 - 6 h. Two hours before the termination of the culture, 50 µL of ethidium bromide is also incorporated to prevent further contraction of chromosomes. Harvesting procedure including treatment using hypotonic solution and fixing cells is done the same way as described earlier for conventional culture. This procedure provides a high yield of late-prophase/early metaphase plates which are most suitable for high-resolution G- banding.

Conventional Giemsa Staining and Chromosome Banding Techniques

Giemsa Staining

The following day after the cells harvested from a conventional or synchronised blood culture, the cells pellet is re-suspended in a fresh fixative (0.5 to 1 mL depending on the quantity of the pellet). Drops of the cell suspension are made on slides, previously cleansed with ethanol and immersed in distilled water and kept at 4ºC. After drops are spreaded and dried in air, they are stained with 5% Giemsa solution in PBS for 3 min. Then, the slides are washed under running tap water and air-dried and examined under a microscope. Cultures with good mitotic index of metaphase or prometaphase chromosomes are identified and used for analyses of chromosome number, morphology and construction of conventional karyotypes. Conventional staining results in uniformly stained chromosomes that differ in size and shape. However, this method is not good enough to differentiate the chromosomes of different species like cattle and goats as they present similar shape and size. This is overcome by applying banding techniques based on elongated chromosomes obtained by reducing amount of colcemid added and treatment time (Iannuzzi & Beradino, 2008).

Most screening programmes for chromosome abnormalities which have been conducted in cattle especially in bulls intended for AI purposes used a simple Giemsa staining method. This has been applied mainly in European countries with the aim of controlling and eradication of ROB (1;29). However, the use of Giemsa staining technique, although it can detect ROB and chimerism for sex chromosome easily but it is less adequate to screen chromosome rearrangements like translocations of reciprocal type which involve the exchange of chromosome segments between non-homologous chromosomes. Hence, it is important to apply more advanced techniques such as high resolution G-banding (GTG) in order to avoid failure of detection and underestimation of chromosome abnormalities that cannot be detected using Giemsa as it is appeared to be for RCT in cattle.

Chromosome Banding Techniques

Chromosome banding is the process of eliciting differences in staining manifestation along the length of a chromosome thereby producing a specific banding pattern. These banding patterns are reproducible, distinctive, and are characteristics to each chromosome. They permit the identification of chromosomes, the construction of karyotypes, detailed description of structural rearrangements, and the comparison of chromosomes within a species or between closely or distantly related species (Hayes, 2000). Based on banding techniques, common chromosome banding nomenclature for bovids has been established (ISCNDB, 2000). The most common banding techniques include G-, R- and C-banding (Sumner, 1990).

GTG-Banding (G-banding by treatment of Trypsin and Giemsa staining)

G-banding involves digestion of less condensed chromatin regions by trypsin, thus reducing affinity to the Giemsa stain, resulting in G-negative bands. In contrast, highly condensed regions, which are less affected by trypsin, result in a more intense Giemsa stain (G-positive bands). There are several G-banding techniques. Slides prepared using elongated prometaphase chromosome spreads stored at room temperature are suitable for G-banding. Trypsin working solution is prepared fresh every time. The concentration of trypsin used might vary from 0.025% to 0.25% in PBS. In our laboratory, 0.25% offered consistent result for cattle. The procedure involves treatment of the slides first with 2xSSC solution in a water bath for 2 min at 60^{o}C. This is followed by dipping the slides in a freshly prepared trypsin solution for 2 min at room temperature. Immerse the slides in normal saline solution for 3 minutes and then, stain with 5% Giemsa in PBS for 10 minutes. Finally, the slides are rinsed with tap water and air dried prior to examination under a microscope for further morphological analysis and construction of karyotypes. The optimum time of treatment at each step should be checked. The procedure described here is modified from Gallimore and Richardson

(1973) and used in our laboratory (Yimer, 2011).

The banding technique such as high resolution G-banding in cattle has been used as a compliment technique for further detail information once abnormality is detected using Giemsa method and for establishment of standard chromosome karyotype (Iannuzzi, 1996). As it is costly and labour-some, its application for routine screening programme was limited in cattle. However, GTG has been used effectively for routine screening programmes for detection of RCT in pigs, in which RCT was found as the most common abnormality. The application of GTG in pigs is economically worthy considering the significant reduction of litter size in carriers of RCT. Moreover, GTG was applied in the establishment of standard normal chromosome nomenclature of a number of species of animals including goats (Iannuzzi *et al*., 1994) and buffalo (Iannuzzi *et al*., 1990).

Constitutive Heterochromatin (C-) Banding Technique

C-banding (constitutive heterochromatin banding) is the best technique for identifications of sex chromosomes and associated abnormalities as they present different pattern from autosomes. The technique involves the use of barium hydroxide to denature repetitive and non-repetitive DNA. When DNA is renatured with 2xSSC, the repetitive DNA sequences renature faster than the non-repetitive ones, visualizing centromeres made up by repetitive sequences.

Slides obtained from both normal and BrdU-treated cell cultures and stored at room temperature for two or more days can be used. Though there are different ways of C-banding, a procedure modified from Sumner (1972) is described in this paper and resulted repeatable result for cattle chromosomes in our laboratory (Yimer, 2011). Initially treat slides with 20% hydrogen peroxide (H_2O_2) in a water bath at 65^{o}C for 3 min. Then, rinse in normal saline at 65^{o}C for 1 min, followed by incubation in 5% $Ba(OH)_2$ solution at 65^{o}C for 3 min and rinsing under tap water. Following a rinse in distilled water and dipping in normal saline for 30 seconds, the slides are incubated in 2xSSC solution at 65^{o}C for 3 min. Finally, rinse the slides in mixture of normal saline and phosphate buffer and stain with 5% Giemsa for 20 minutes. After drying in air, the slides are examined under a microscope.

Fluorescent in Situ Hybridization (FISH) Technique

It is an advanced tool in the field of cytogenetics and has been applied as a classical technique in human cytogenetics. The technique is based on visualization of fluorescent hybridization signals present on specific chromosome regions (bands). This can be done by using DNA-probes containing fluorochromes conjugated with compounds that recognise the labelled DNA-probes. It is useful for precise identification of structural chromosome abnormalities and to study complex chromosome aberrations. Its application in animal cytogenetics is however limited by lack of commercial

availability of molecular probes unlike humans. Moreover, its application in animal cytogenetics for routine screening programme may not be economically worthy. A more detail description of the blood cell culture, banding techniques and FISH can be referred to the publication by Iannuzzi and Beradino (2008).

CONCLUSION

The importance of cytogenetic screening, as a part of a preventive measure for infertility problems associated with chromosome anomalies in a modern breeding system, has been very clear since Gustavsson has demonstrated the correlation between ROB (1;29) and reduced fertility in Swedish cattle in 1979. Abnormal chromosomal karyotypes lower reproductive performance of farm animals through decreasing ability or complete failure to produce functional gametes and death of embryos. Thus, it is quite important to extend cytogenetic investigations in farm animals including cattle breeds in the tropics to obtain a base line data on occurrence and significance of chromosomal anomalies that will lead to what strategies to be designed accordingly. Moreover, establishment of regulatory and monitoring systems to make sure animals (local or imported) intended for breeding are screened against cytogenetic anomalies would benefit the farming industry by minimizing associated reproductive failure. For more effective cytogenetic investigations, there is a need for integration among already established cytogenetic laboratories, more collaboration with breeders, veterinarians and animal scientists, as well as creating awareness among students leaving veterinary schools and animal health workers at farm level. Although the use of FISH technique with molecular markers offered great advantages for unambiguous identification of chromosomes and chromosome regions involved in chromosome abnormalities, its application is limited as it is costly and because of lack of commercial availability of chromosome specific probes. Nevertheless, the use of conventional Giemsa staining and banding techniques might be good enough to obtain repetitive and satisfactory results in cytogenetic investigations of common problems.

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