Overview of Clinical Cytogenetics in Mammalian Livestock

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The School of Biosciences

Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or institutions of learning.

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I would like to give a huge thanks to my supervisor, Darren Griffin for accepting me on to the project and for his support throughout the year. I would also like to give thanks to Carla Canedo-Ribeiro, Lisa Bosman and Giuseppe Silvestri who together gave endless support throughout my master's and have helped me develop as a scientist.

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Table 4.5: Images on the left-hand column show how probes normally attach to the chromosomes of the control (cattle). Images in the right column show the affinity of hybridisation of the same probes attached instead to sheep chromosomes. The position of probes on each chromosome is also shown. This figure shows how probes for bovine chromosomes 20, 21, 22, 23, 24, 25, 29 and X bind to a control cattle sample (left) and an ovine sample (right).

Abbreviations

COVID- Corona Virus Disease

DNA- Deoxyribonucleic acid

FISH- Fluorescent *in situ* Hybridisation

MBG H_2O- Molecular biology grade water

NOR- Nucleolus organizer regions

RobT- Robertsonian translocation

RT- Reciprocal translocation

rDNA- Ribosomal DNA

SMC- Structural maintenance of chromosomes

Abstract

Whilst balanced chromosomal aberrations, often do not present phenotypically in carriers they are known to cause reduced fertility in livestock. Mammalian livestock are of vital economic importance with pigs and cattle providing over 50% of consumed meat globally. Furthermore, cattle are the main providers of a dairy industry worth over $750 billion. In an industry where a small population of high value males (boars/bulls/rams) are used in the vast majority of artificial insemination, the potential financial loss caused by a sub-fertile male could be considerable especially if aberrations are passed down to offspring. This thesis reports on recent data collected using novel Multiprobe® Fluorescent *in situ* Hybridisation (FISH) devices on porcine and bovine blood samples for robust systematic screening of chromosomal abnormalities. There is significant potential for widespread use of these devices as they offer a more sensitive and accurate approach than given with previous methods such as karyotyping. Using this method 775 pigs and 3 cattle were screened. We reported that 14.5% of screened pigs presented with 8 different types of abnormality which is a far higher prevalence then previously recorded by karyotyping. 2 of 3 cattle samples also presented with an abnormality. Our results showed that poor quality of many samples received in 2021-2022 due to delivery complications meant that translocations were undetectable using a standard karyotype. However, FISH works well even with sub-optimal sample quality meaning time and money would not be lost in the repeating of sample collection and delivery. Multiprobe® device analysis is a comprehensive, robust system in bovine and porcine samples therefore, there is potential for it to work in other livestock species. This thesis reports on the use of probes selected for the bovine Multiprobe® device to test the extend they will bind to ovine (sheep) chromosomes to potentially create a novel ovine Multiprobe® device.

1: Introduction

1.1: Background

Global meat production is three times higher than it was 50 years ago being in large part down to a 2.5 fold increase in the world population(1). Pig meat is the most popular red meat accounting for approximately a third of all produced meat, with production rising 4-5 fold in the last 60 years from 25 million tonnes in 1961 to 110 million tonnes in 2020(2). Beef production from cattle has risen approximately 2.5 fold since 1961 and represents 21% of the total meat produced(2). Dairy cattle are also the main source of milk worldwide and is the main provider of a worldwide dairy industry worth $750 billion in 2019(2, 3). Sheep and goats are also significant providers of meat worldwide with combined meat production rising from 6 million tonnes in 1961, to 16 million tonnes in 2020. Sheep and goats also offer an alternative source to cattle milk and other dairy products. Sheep also have significant use in wool production(2, 4).

Artificial insemination (AI) is now the predominant method of breeding in both the domestic pig and cattle (especially in the dairy industry) while also being routine in sheep and goats(5-7). Historically, breeding males have been picked for maximum productivity whether that is higher meat yields or having female offspring which are superior producers of milk. As fertility of individuals has been less of a consideration, the prevalence of sub-fertile individuals in the small pool of breeding males is ever increasing(5). One of the main causes of subfertility in livestock is the presence of a chromosomal aberration(8). As chromosomal aberrations are a relatively common occurrence in cattle and pigs, there is a growing demand for the chromosomal screening of these issues in boars or bulls prior to semen extraction. As one breeding bull can inseminate up to 130,000 to 200,000 cows in its lifetime, optimum fertility is

paramount to prevent significant financial loss(5). Screening for chromosomal abnormalities can be achieved with high rates of accuracy through cytogenetic techniques such as Karyotyping and Fluorescent *in situ* Hybridisation (FISH).

Both karyotyping and FISH involve the culture and harvesting of a cell or tissue sample, subsequent mounting of this sample onto a slide and application of a fluorescent DNA binding dye, for visualisation of chromosomes under a microscope(5). The key differences are that in karyotyping, mounted chromosomes are often dyed with specialised stains which create a banding pattern between the denser and less dense regions of the chromosomes to help differentiate between non-homologous chromosomes. Subsequent images are taken, and individual chromosomes are ordered in a diagram so that any irregularities can be located. In the context of locating chromosomal aberrations, FISH involves attachment of fluorescent probes to specific target regions of each chromosome, in the case of this thesis specifically to the sub-telomeres (near the end of chromosomes). Sub-telomeric probes are used due to these regions being at the end of the chromosome meaning any translocation, no matter how small should cause the probe target sequence to be on the translocated region. This allows a scientist to systematically check each chromosome in the target animals genome for the presence of the target fluorescent probes. If probes are in abnormal positions this will likely suggest an aberration in the individual(5, 6).

This thesis will focus on the importance of cytogenetic diagnosis of chromosomal abnormalities, and how FISH offers a better alternative to the more traditional method of karyotyping. This is due to a higher sensitivity and a higher rate of successful diagnosis. This thesis will also test the potential of using cattle diagnostic probes on

ovine (sheep) chromosomes for the potential later production of a sheep diagnostic device.

1.2: Genomics and Cytogenetics

1.2.1 Chromosome basics

Chromosomes are long DNA molecules that contain part of (or all of) the genetic material of an organism (9). The genome of a eukaryotic organism can be found within the set of chromosomes within a single nucleus. In animals, somatic cells are diploid meaning they contain two sister copies of each chromosome (one from each parent of the individual), excluding the sex-determining chromosomes. Every gene found on a single chromosome has a duplicate gene found on the sister chromosome(9, 10). During interphase chromosomes decondense/unravel into chromatin. Chromatin is made up of repeating units called nucleosomes (11) consisting of a segment of DNA wrapped around a core histone octamer complex. The histone octamer core is made up of 8 histone molecules: 2 each of H2A, H2B, H3 and H4(12). Positive charges on histones bind to negative charges on DNA in a specific conformation which allows a segment of DNA ~150bp long, to wrap around each histone octamer just under two times. Nucleosomes stack closely together into organised arrays with multiple levels of packaging(9-12). The first level of packaging produces a fibre 30nm wide. This fibre folds back on itself repeatedly creating a series of loops which compact together allowing a large amount of DNA to occupy a small amount of space (9,11).

Chromatin can be further classified into heterochromatin where nucleosomes are packaged closer together making the chromatin more densely packed and euchromatin which is less densely packed (9-12). Banding stains (e.g., C-Banding, Giemsa-banding) used in Karyotypes often stain euchromatin one colour and

heterochromatin another colour creating a banding pattern characteristic of each chromosome.

1.2.2 Mitosis and chromosome condensation

The cell cycle includes production of a new cell, growth and development into a mother cell and division into two identical daughter cells. The division of a mother cell firstly requires appropriate replication of all the mother cells organelles and genetic material (chromosomes). The two main phases of the cell cycle in eukaryotic cells are: Interphase where all the genetic material is replicated and mitosis where the diploid set of chromosomes divide at the centromere separating two equal sets of sister chromatids into a newly forming daughter cell(13, 14). Interphase is further split into 3 stages: The Gap 1 (G1) phase, where the cell replicates is organelles and grows in size. The Synthesis (S) phase, where the cell replicates its DNA and chromosomes are highly unravelled in the nuclear envelope to promote replication. The Gap 2 (G2) phase, where the cell continues to grow until all the necessary organelles for two daughter cells are produces ready for the mitotic phase (14). After interphase each chromosome consists of two equal sister chromatids connected by the centromere. The mitotic phase is split into distinct stages:

- **Prophase:** Condensation of homologous chromosomes is mediated by proteins such as Topoisomerase II which may assist condensation by its decatenating activity on chromosomes (15). SMC including condensin and cohesin promote tight looping of chromatin creating chromatin fibres within individual chromatids and mediate cohesion between sister chromatids. The proposed mechanism for condensin/cohesin condensation of chromatin is through loop extrusion where condensin binds to a small loop of DNA and

subsequently expands this loop until neighbouring loop-extruding complexes converge (Figure 1.1). This process explains condensation of chromatin into small chromosomes by organisation of DNA into a series of loops around an axial core containing condensin(16, 17). Finally, phosphorylation of histone proteins H3 and H2A may have a role in the condensation of chromosomes(18). Negative ends of microtubules emanating from the centrioles within centrosomes attach to opposing poles across the cell.

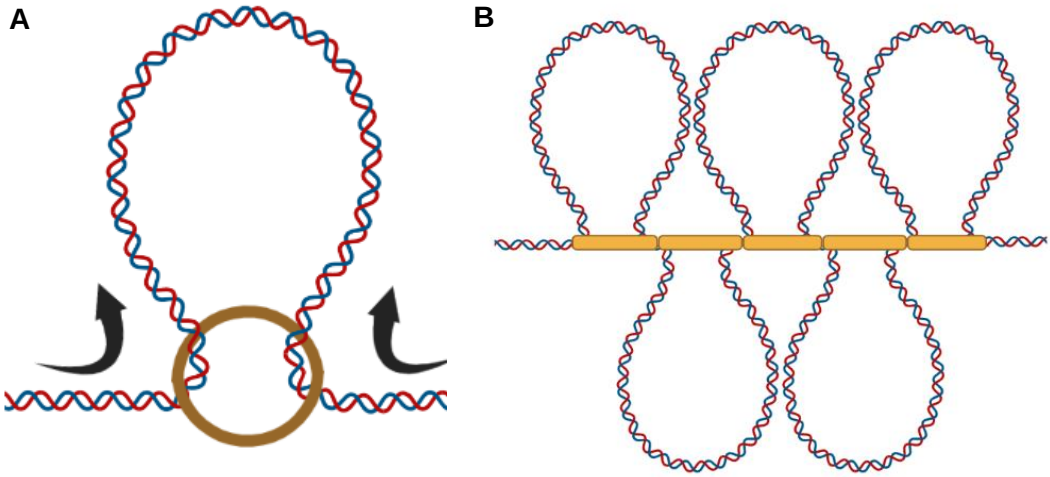


Figure 1.1: **A:** Loop extrusion by condensin (brown circle) **B**: Axial core of adjacent condensins creating a series of closely interacting loop-extruding complexes.

- **Prometaphase:** In prometaphase disassembly of the nuclear envelope is triggered by cyclin-dependant kinase. + ends of microtubules emanating from the centrosomes attach to kinetochores within the centromeres of each chromosome(19).
- **Metaphase:** Chromosomes align in a plain across the cells equator attached to microtubules on the kinetochores.

- **Anaphase:** Microtubules retract, separating sister chromatids attached to respective kinetochores in centromeres of each chromosome. Sister chromatids of each chromosome are pulled to opposing sides of the cell.
- **Telophase:** Sister chromatids reach opposing poles of the cell. Cytokinesis begins where the plasma membrane cell starts to divide around equator region. Sister chromatids now considered separate chromosomes, begin to decondense. The nuclear envelope also reforms.
- **Cytokinesis:** Cells divide creating two new interphases with decondensed chromosomes (20).

The mitotic index is the ratio of cells undergoing mitosis to the total number of cells in a population. Chromosomal aberration screening requires a high mitotic index, as chromosomes are not visible when cells are in interphase due to obstruction from the nuclear envelope and chromosomes being unravelled. Chromosome analysis typically further requires for cells to be in the prophase-metaphases stages of the cell cycle so that the chromosomes are condensed, contain both sister chromatids and are visible under a light microscope. However, as 95% of the cell cycle is spent in interphase the mitotic index is usually very low(20).

Ideal chromosome analysis requires culturing of cells for an optimum period of time whereby a maximum number of cells are likely to be undertaking mitosis. The mitotic index may also be increased by treating cells for a short period before cell harvesting using compounds which inhibit stages in the cell cycle. The most commonly used example of this is the mitotic inhibitor Colcemid. Colcemid binds to tubulin, leading to rapid microtubule disaggregation, preventing chromosomal segregation, and therefore inhibiting mitosis. As Colcemid prevents cells from proceeding past metaphase it is

ideal for chromosomal analysis as it leads to a clear, condensed, diploid set of chromosomes(21).

1.2.3 Gametogenesis

Gametogenesis is the production of gametes from the germ line. Every female or male animal have germ line tissue, with individual cells in this tissue are referred to as germ cells. These cells undergo meiosis to produce 4 equal haploid precursor cells which then proceed to directly develop into gametes. In animals, two distinct differentiation programmes produce two morphologically different types of gametes (male or female) (22).

The stages of meiosis are as follows:

- **Prophase I:** Synapsis of homologous chromosomes occur whereby homologous chromosomes come together to form a tetrad or bivalent which contains 4 sister chromatids. Recombination occurs between any two chromatids within a tetrad structure. The nuclear envelope dissolves and meiotic spindles begin to emanate from centrioles.
- **Metaphase I:** Paired homologous chromosomes line up across the cell equator. Centrioles complete migration to opposing poles of the cell and spindles attach to the centromere of one of each pair of chromosomes.
- **Anaphase I:** Homologous chromosomes are pulled to opposing poles of the cell. Sister chromatids remain attached in contrast to mitosis where sister chromatids are separated to separate poles of the cell.

- **Telophase I:** On each opposite end of the cell is a haploid set of chromosomes which decondenses. A new nuclear envelope forms and the cell undergoes cytokinesis.
- **Prophase II:** The haploid set of chromosomes condense. Centrioles once again replicate, and spindles begin formation. The nuclear membrane brakes down.
- **Metaphase II:** The chromosomes begin to line up along the cell equator end to end. Spindles from centrioles once again attach to kinetochores in the centromere of each chromosome.
- **Anaphase II:** Sister chromatids are pulled to opposite poles of the cell.
- **Telophase II:** A nucleus forms around each set of sister chromatids, now considered a complete haploid set of chromosomes. Cytokinesis occurs (9, 20, 22).

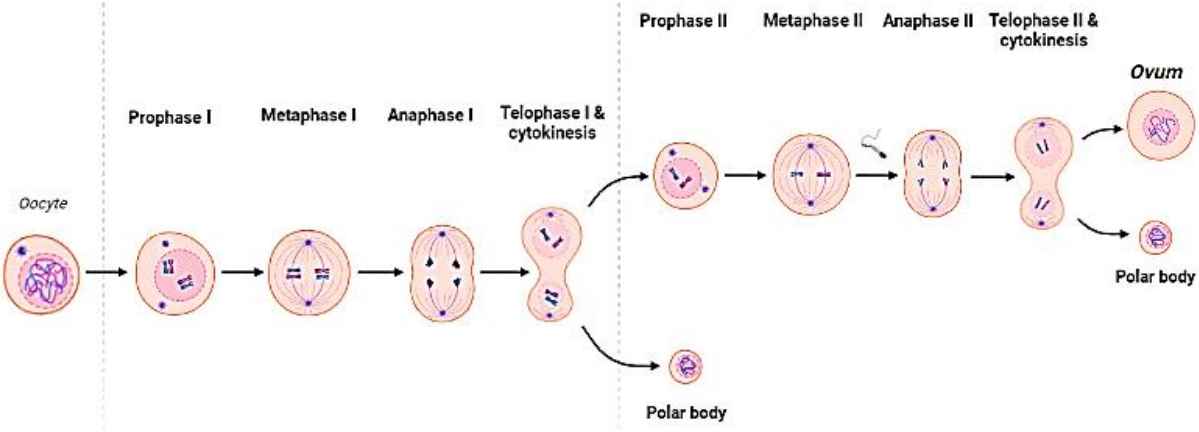


Figure 1.2: Created with Biorender.com. Gametogenesis of a female gamete from a germ line cell.

In mammalian males meiosis creates four precursor haploid cells which each form a sperm cell. However, in females telophase I of the primary oocyte (haploid precursor) produces one large cell and once small cell. The larger cell continues meiosis normally and after telophase II the cell once again produces one cell far larger than the other.

The two smaller cells are known as known as a polar body and have no significant function after meiosis. Finally, the resultant large cell becomes an ovum (egg cell)(figure 1.2) (23).

1.2.4 Chromosome configurations/morphologies

While analysing chromosomes for aberrations using karyotyping or FISH, chromosomes would preferably have to be in the metaphase stage of the cell cycle. A whole set of chromosomes grouped together in this stage is thus termed a metaphase. It is in this configuration where the characteristic X-shaped chromosomes are seen under light microscope. However, this is not the only common shape in mammalian chromosomes, with several other morphologies in this phase of the cell cycle (figure 1.3). A single chromosome within a metaphase consists of two equal sister chromatids joined together by a centromere where chromosomes are constricted. The centromere divides each sister chromatid into two ‘arms’ either side of the centromere. The shorter of the two arms is always termed the p arm while the other is termed the q arm.

1. **Metacentric:** This is where the centromere is at the centre of the chromosome and the p and q arms are the same length.

2. **Submetacentric:** The centromere is identifiability off centre causing the q arm to be longer than the p arm.

3. **Acrocentric:** The centromere is distant from the centre of the chromosome and the q arm is significantly longer than the p arm.

4. **Telocentric:** the centromere is near the end of the chromosome causing the p arm to be barely visible under a light microscope.

Figure 1.3: **Created with *Biorender.com*** *Possible conformations of mammalian chromosomes in metaphase***.**(24)

These conformations (figure 1.3) play an important role in cytogenetics particularly in karyotyping as they are a major structural element of chromosomes which assists in differentiating chromosomes from one another, in order to label each chromosome with a specific chromosome number.

1.3: Chromosomal abnormalities

1.3.1 Reciprocal translocations

Reciprocal translocations (RTs) are the result of a DNA segment of one chromosome being exchanged with a DNA segment of another non-homologous chromosome with no loss or gain of DNA at the breakpoint thus making it a balanced rearrangement (figure 1.4). Individuals with a balanced reciprocal translocation are most commonly phenotypically normal unless the translocation breakpoint is within a dominant coding gene, or the rearrangement is in close enough proximity to coding genes to cause decreased expression (25).

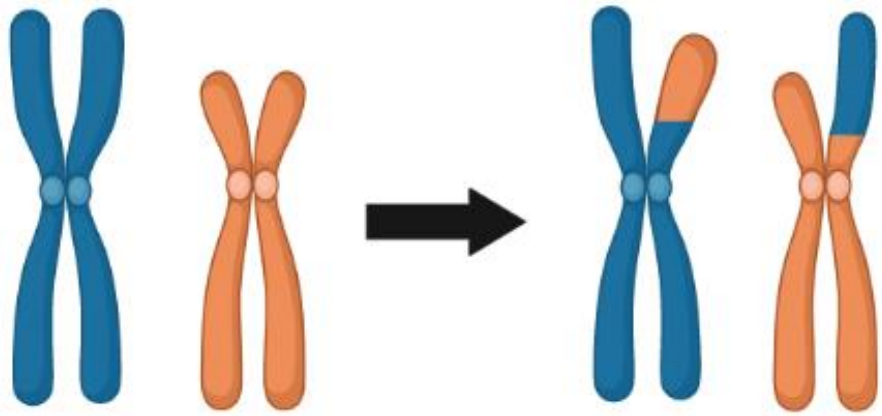


Figure 1.4: Created with Biorender.com, Non-homologous chromosomes before and after reciprocal translocation.

Although reciprocal translocations may not cause many phenotypic changes, they are very likely to cause some level of subfertility in carriers. Autosome-autosome translocations, have to create a pairing cross (quadrivalent) between translocated chromosomes and their normal homologues in order to progress through meiosis I.

This mediates infertility in several ways:

First, anaphase I of quadrivalent structure creates balanced and unbalanced gametes. Segregation occurs in 5 possible modes: alternate, adjacent-1, adjacent-2, 3:1 and 4:0. Alternate segregation results in balanced gametes; all other possible outcomes produce unbalanced gametes. Alternative, adjacent-1 and adjacent-2 all produce 2:2 gametes meaning out of the 4 chromosomes involved in the quadrivalent, 2 will segregate into each cell. These outcomes are visualised in figure 1.5.

[REDACTED]

Figure 1.5: This diagram shows possible modes of segregation for a quadrivalent structure. This excludes 4:0 where all chromosomes to one cell leaving the other cell with none of the chromosomes involved in the quadrivalent. *Chromosome Abnormalities and Genetic Counseling , 3rd Edition***,** 4. Autosomal Reciprocal Translocations"(48).

The mode in which segregation will occur is dependent on a number of factors such as the size of the translocated regions, the size and structure of the chromosomes effected, the parents gender, the gene content, and the position of the translocation breakpoints in each patient. Unbalanced gametes are rarely compatible with live and will therefore rarely be able to fertilise an ovum.(25, 26) Second, the mechanisms and time constraints of the formation and later segregation of this structure may hinder the meiotic process(26). Third, asynaptic regions between chromosomes in the quadrivalent structure are common leading to failed meiosis and death of the effected germ cell. Fourth, there is evidence to suggest that translocated segments bind to non-homologous X and Y chromosomes, preventing X inactivation. This has a gene dosage effect, eliminating the germ cell(27). Finally, interactions between the cell nucleus and translocations may hinder meiosis and thus cause cell death(28). Due to these mechanisms non-disjunction in meiosis may occur leading to aneuploid gametes.

1.3.2 Robertsonian Translocations

A Robertsonian translocations is a fusion between two chromosomes which can be homologous or non-homologous and is the most common translocation in cattle (29).

This translocation is caused by the breakage of two acrocentric chromosomes in the centromeric region and fusion of the respective parts of the two acrocentric chromosomes with the parts of the other chromosome. This creates two new chromosomes one containing the larger q arms and the other containing the two shorter p arms fused together which, due to their miniscule size usually disappear within a few cell divisions (figure 1.6). Robertsonian translocations can either be monocentric where there is one centromere present or dicentric where the new chromosome has two centromeres. In cattle with the exception of the very common translocation Rob(1;29), almost all other translocations which have been found have been dicentric(29).

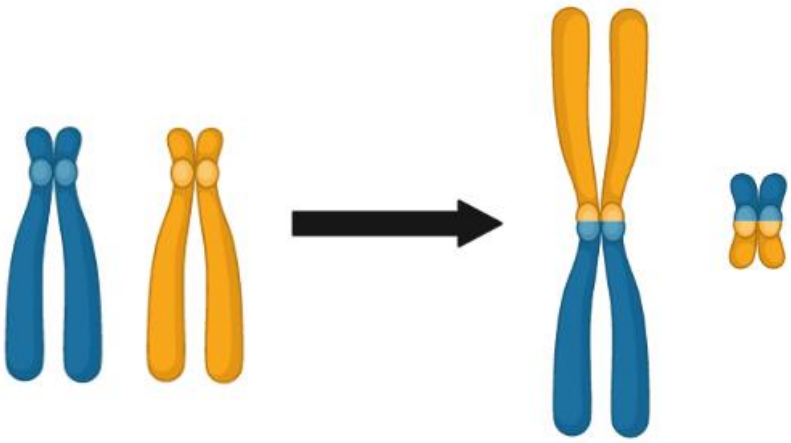


Figure 1.6: Created with Biorender.com, Acrocentric chromosomes before and after occurrence of Robertsonian translocation.

The loss of the shorter p-arms often does not result in significant gene-loss as this region commonly only contain few non-essential repeats and repeated code. Thus, Robertsonian fusions are usually considered balanced translocations and carriers are often phenotypically normal however, some translocations may result in unbalanced chromosomes. The fusion results in the net loss of one chromosome. The majority of Robertsonian fusions are thought to be influenced by the presence of nucleolar organizer regions (NORs) which contain genes coding for ribosomal RNA (rDNA) (30). During meiosis a carrier cell may create gametes with too few or too many chromosomes (figure 1.7). When fertilized these gametes produce aneuploid embryos which in the vast majority of cases result in embryonic mortality or otherwise severe issues in development(31).

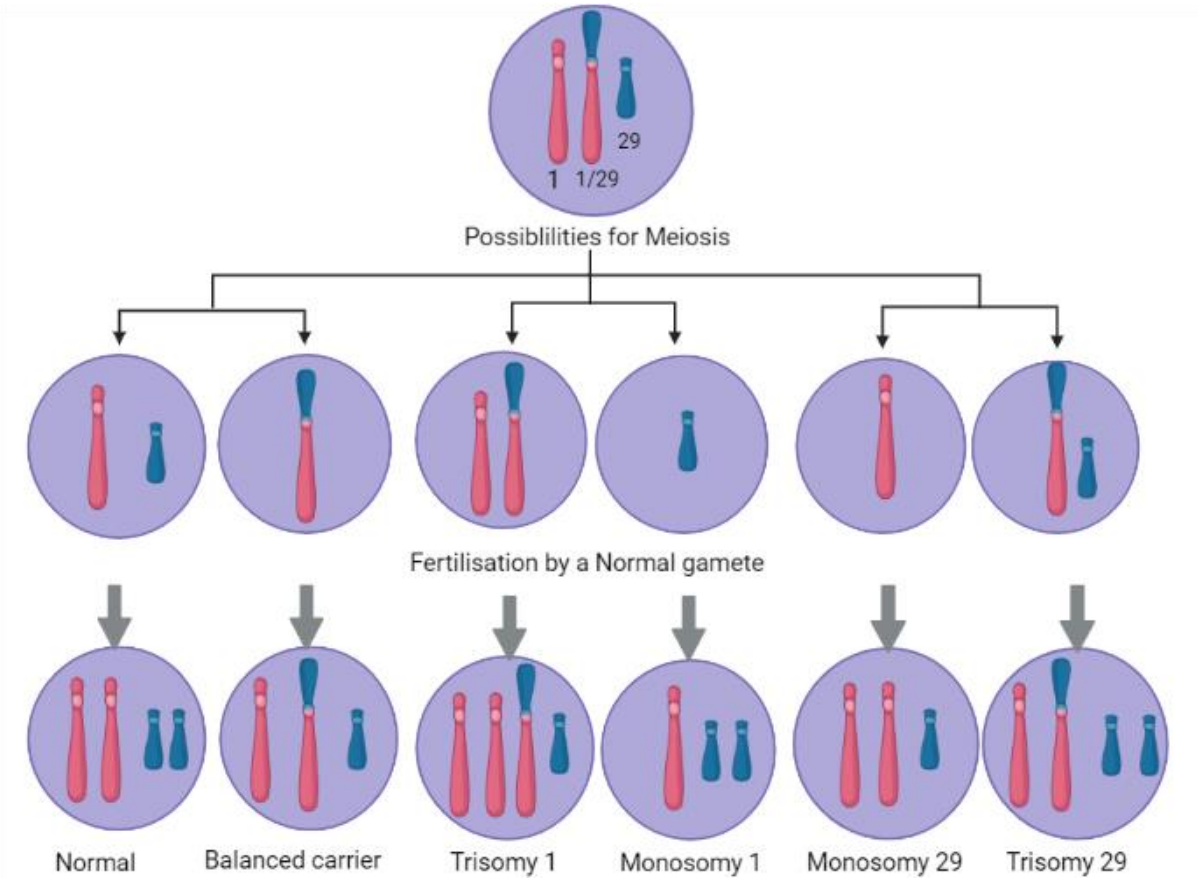


Figure 1.7: Created with Biorender. Possible outcomes of a Robertsonian 1/29 post meiosis and fertilisation of each meiotic cell with a normal gamete.

In cattle 44 different Robertsonian translocations have been recorded up to 2015. By far the most commonly occurring amongst these is the RobT(1:29) translocation. This translocation has been identified with 50 different cattle breeds worldwide and has been found with a prevalence of up to 60% in British White (32) and Corsican breeds(33). Monosomic (-1 chromosome) and trisomic (+1 chromosome) embryos have been detected in earlier studies involving chromosome analysis of embryos extracted from cows inseminated with semen of heterozygous carriers of RobT(14;20) and RobT(1;29). However, very few living calves with autosomal monosomy or trisomy have been found therefore suggesting prenatal death of these offspring(34). With such a high prevalence in cattle, Robertsonian fusions in particular RobT(1;29) likely have a significant economic burden when looked at on a larger scale. RobT(1;29) alone causes a reduction in fertility of 3-5% which could be detrimental if not located, given a single bull often is used to inseminate 100,000-200,0000 cows (35, 36).

1.3.3 Aneuploidy

Aneuploidy is a condition whereby a member of a species has an abnormal number of chromosomes. Aneuploidy is a major cause of prenatal death in mammalian livestock and likely has an underestimated impact on successful insemination as chromosomal analysis of eliminated embryos and still-births rarely occurs. Aneuploidy primarily arises in maternal germ cells from nondisjunction between homologous chromosomes during meiosis I. Although, this is not always the case with aneuploidy also arising from errors in paternal or meiosis II. In both porcine and bovine chromosomes, non-disjunction occurrence increases during meiosis of oocytes in older females, increasing the likelihood of aneuploidy(37, 38). This is known as the ‘maternal age effect’. Several exogenous and endogenous factors contribute to increasing rates of aneuploidy in increasing maternal age such as, recombination

failure, cohesin deterioration, spindle assembly checkpoint (SAC), dysregulation, abnormalities in post-translational modification of tubulins ad histones, and mitochondrial dysfunction (49). In humans this effect is well documented with increasing rates of trisomy 21 (Down's Syndrome) births, with increasingly older mothers.

Preimplantation genetic testing for aneuploidy (PGT-A) has revealed not only that aneuploidy is common in cattle but also that incidence of successful clinical pregnancy in aneuploid embryos is only at 5.8%(39). Aneuploidy has been recorded in liveborn but most commonly results in extreme anatomical malformations/defects, infertility and in most cases early death (recently reviewed by 40). Aneuploidy in the sex chromosomes is far more common and results in far greater viability, however, still has a negative effect on fertility(41).

Porcine aneuploid embryos have also been detected(42). However, similar to cattle incidence of live pigs with autosomal aneuploidy has rarely been reported despite decades of testing suggesting early prenatal death of offspring. A few cases of pigs with aneuploidy in the sex chromosomes have however been reported(43, 44).

1.3.4 Mosaicism/Chimerism

Mosaicism is a condition whereby two or more chromosomally distinct cell populations exist within a single organism. One of the primary mechanisms in which this occurs is through a post-zygotic error. An example of this is non-disjunction in a replicating cell within a blastocyst which causes aneuploidy in a relatively small proportion of cells depending primarily on how early in blastocyst development the error occurred. Mosaicism can also occur through meiotic non-disjunction and subsequent "embryo correction" or "trisomy rescue" of some blastocyst cells; the rates of which may be

increased in older maternal individuals. In this scenario >50% of cells will likely be aneuploid(50, 51, 52). Depending on which chromosomes are affected and the proportion of aneuploid cells, mosaicism can cause varying degrees of sub-fertility in carriers and therefore could pose a significant economic loss when not identified in individuals (50, 51, 52).

A chimera (freemartin) is a eukaryotic organism which again has more than one distinct genotype in different cell populations(53). Unlike mosaicism the chromosome number in each cell population should be normal however, these chromosomes from the other genetic population are a random new daughter set of genes from the parents and are therefore completely unidentical to the other population within the organism. Animal chimerism is the result or the merger of two (or more) embryos each containing a unique set of genetic information(54, 55). The resulting merged embryo can develop into a viable adult with two or more sets of DNA. This may result in a XX/XY intersex individual with both female and male genitalia which increases risk for infertility (53, 54, 55).

1.4: Cytogenetic methods

1.4.1 Karyotyping

Karyotyping is a cytogenetic tool whereby a specialised scientist will culture and harvest an individual's cells, mount them on a slide and stain them in a banding pattern for visualisation under a microscope. An image of the individuals chromosomes from one cell in the prophase-metaphase stages of the cell cycle is then taken, and each chromosome is cut out individually and assigned a number to distinguish specific chromosomes. Homologous chromosomes have identical gene content and are structured identically to one another and are therefore grouped together. Chromosome numbering is determined by size, banding patterns and centromeric positioning (e.g., metacentric/acrocentric). The most common banding stain used in karyotyping is the Giemsa-banding stain. Abnormalities in chromosomes are much easier to find in a karyotype then simply visualising a metaphase as chromosomes are lined up and can be compared to one another (56).

The domestic pig has a relatively small number of chromosomes at 38 diploid (19 haploid). These chromosomes are relatively varied in morphology and size making karyotyping relatively easy (figure 1.8). The X chromosome is a medium sized metacentric chromosome (57).

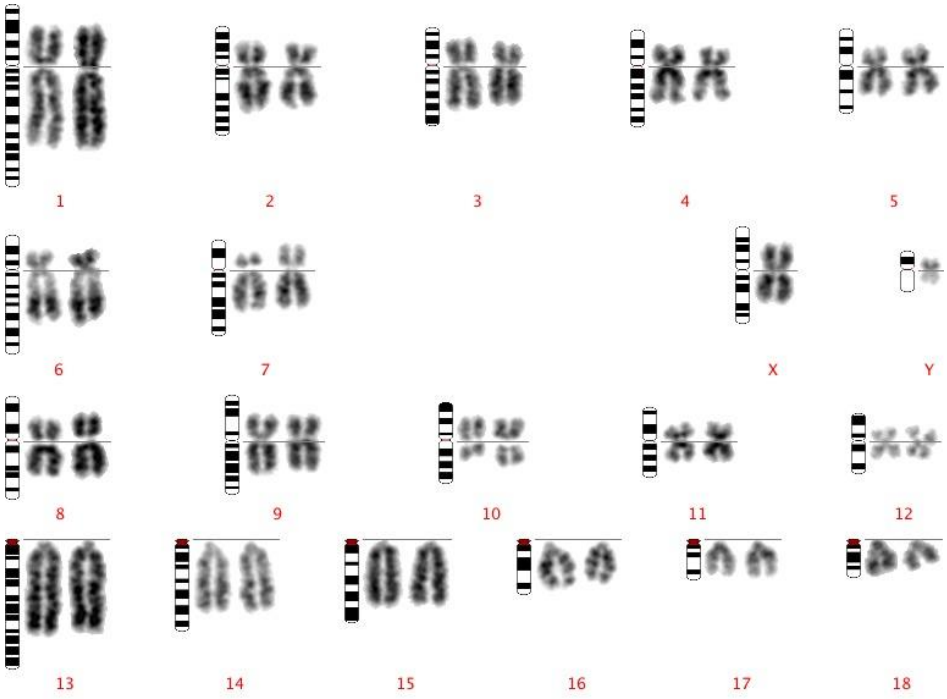


Figure 1.8: Standard karyotype of a normal boar (*sus domesticus*).

Cattle have an unusual karyotype as all autosomes are acrocentric and have less size variance compared with porcine chromosomes. Cattle have a much larger haploid chromosome number of 30 (diploid 60) and have a submetacentric X chromosome (Figure 1.9). As specific chromosomes are hard to distinguish in cattle due to all autosomes are acrocentric and similar in size, banding is often used to differentiate. Banding commonly involves highlighting the less dense euchromatin either white or black and heterochromatin the opposite. Each chromosome has a unique banding pattern (58).

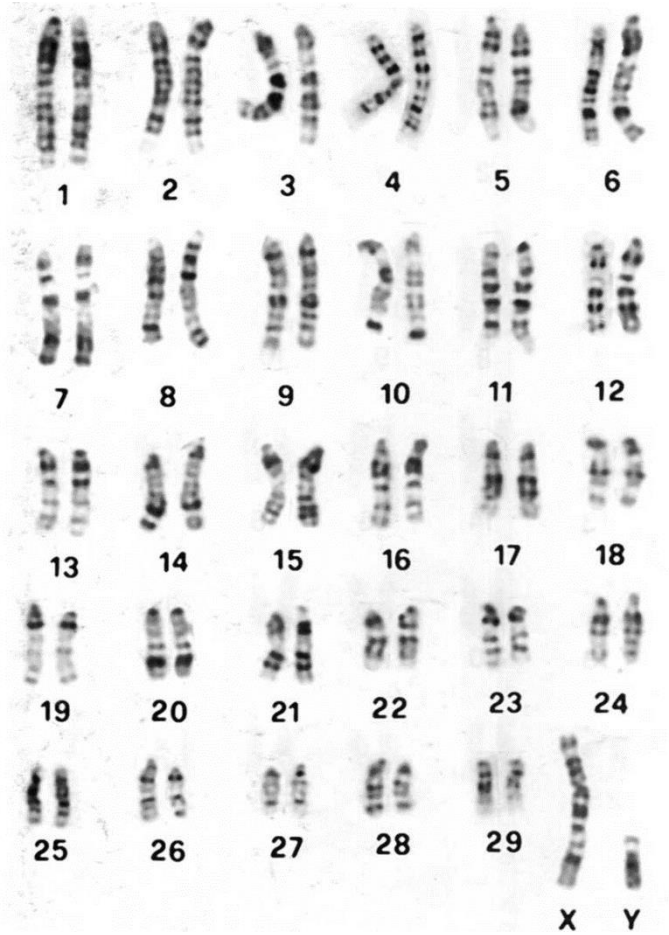


Figure 1.9: Standard banded karyotype of a normal bull (Bos taurus).

The sheep Karyotype contains 3 large submetacentric (1-3) autosomes with the rest (4-26) being smaller acrocentric chromosomes. The X chromosome is also acrocentric (figure 1.10). The haploid chromosome number in a sheep is 27 (56 diploid). There are high amounts of visible homology between sheep and cattle chromosomes. Primarily due to both having almost exclusively acrocentric autosomes. Interestingly, sheep have 3 less homologous pairs of chromosomes then cattle. However, their 3 largest chromosomes are submetacentric (not acrocentric) which may suggest fusions of previously acrocentric chromosomes in a common ancestor. This is supported by data collected in this thesis as probes targeting the smaller and larger autosomes of the cattle chromosome set appeared to bind in the sub-telomeres and centromeric regions of the ovine (sheep) submetacentric chromosomes 1, 2 and 3 (59).

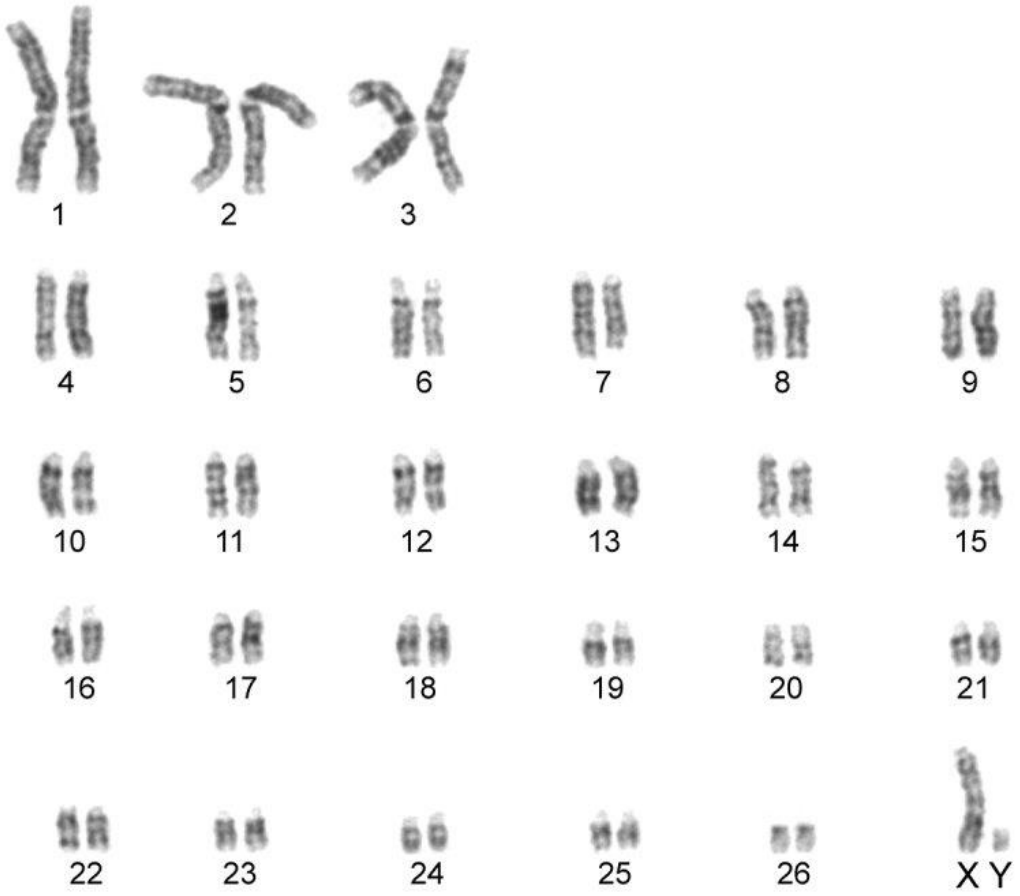


Figure 1.10: Standard banded karyotype of a ram *(Ovis aries)*.

1.4.2 Fluorescent in situ Hybridisation (FISH)

FISH is a cytogenetic tool used for finding spatial genomic and transcriptomic information, by utilising the hybridisation of an endogenous single strand of DNA or RNA with a complementary strand of DNA or RNA labelled with a fluorophore (e.g., Texas Red or FITC) (45). This labelled complementary strand of DNA is termed a probe. In older versions of the *In Situ* Hybridization technique, complementary DNA strands were often radiolabelled i.e. using H_3. Since its discovery, FISH has steadily become one of the most powerful cytogenetic tools for analysing cells and tissues at a genome or transcriptome level (45, 46). One of the most significant examples of this is its growing use in locating all the chromosomal aberrations mentioned above in the livestock breeding industry.

Novel diagnostic Multiprobe® devices have been produced for mammalian livestock firstly in pigs (47) followed by cattle (35) as recently as 2017 and 2020 respectively. With the success of these devices, it is likely that soon a device will be produced for other mammalian livestock. Such a device consists of essentially a piece glass apparatus segmented into regions corresponding to each chromosome of the target species. In each box two probes (p and q) are selected and are inserted onto each glass box. The DNA segment of each probe is complementary to an extremely highly conserved segment of sub-telomeric regions of the p and q arms of the target chromosome (figure 1.11). The chromosomal target must be conserved such that the complementary strand of DNA must work on every individual of the target species. Sequences within sub-telomeric regions are selected from NCBI databases. Bacterial Artificial Clones (BACs) which best fit into these highly conserved regions are selected and ordered. These are subsequently grown and fluorescently labelled converting the

BACs into FISH probes. A specific device is then produced which contains a box corresponding to every chromosome in the target animals genome.

The benefit of such a device is that the selected BACs which are converted into FISH probes bind with very high specificity to target sequences. Moreover, the appearance of bound probes on a target chromosome is very distinctive. Two clear dots will form for each probe either side of the chromosome. If the correct probes appear on the correct chromosomes, it is extremely likely that no translocations have occurred. If probes from one end of a chromosome appear on a non-homologous chromosome than it is almost certain that a translocation has occur between these non-homologous chromosomes. Furthermore, as our Multiprobe® probes bind to the subtelomeric regions which are found very close to the end of the chromosome(only preceded by the telomeres) any translocation which will be large enough to potentially effect fertility will be located even if these translocations don't cause any visible change to chromosomes under a light microscope. This is why FISH is a far superior tool to karyotyping which is poor at detecting subtle, cryptic translocations.

Chr 1 p+q	Chr 2 p+q	Chr 3 p+q	Chr 4 p+q	Chr 5 p+q	Chr 6 p+q	Chr 7 p+q	Chr 8 p+q
Chr 9 p+q	Chr 10 p+q	Chr 11 p+q	Chr 12 p+q	Chr 13 p+q	Chr 14 p+q	Chr 15 p+q	Chr 16 p+q
Chr 17 p+q	Chr 18 p+q	Chr X p+q					

Figure 1.11: Example of the probe content of a FISH Multiprobe® device specifically targeting porcine chromosomes.

1.5: Specific Aims

Specific Aim 1a: Firstly, the application of a porcine Multiprobe® FISH diagnostic device on pig (Sus domesticus) samples to screen for chromosomal aberrations before the use of this pig in AI breeding and to characterise and report on the prevalence of chromosomal abnormalities. Secondly, to determine whether screening could be untaken to the same degree using a simple karyotype.

Specific Aim 1b: Firstly, the application of a novel bovine Multiprobe® FISH diagnostic devices on cattle (Bos taurus) samples to screen for chromosomal aberrations before the use of this cattle in AI breeding and to characterise and report on the prevalence of chromosomal abnormalities. Secondly, to determine whether screening could be untaken to the same degree using a simple karyotype.

Specific aim 2: Testing the extent in which optimised subtelomeric cattle probes, bind to sheep chromosomes (*Ovis aries*) for potential future use in the production of a novel ovine Multiprobe device.

2: Materials and Methods

2.1: Production of the subtelomeric-targeting cattle Multiprobe® device

2.1.1 Growing and aliquoting of BACs embedded in agar stabs

15mL Falcon tubes were filled with 2ml LB broth. A sterile pipette tip was used to take a scraping of a -80^{o}C glycerol stock containing bacterial artificial clones (BACs). The sterile tip was then placed in the Falcon tube containing LB broth and the Falcon tubes were incubated with lids open for 16 hours at 37^{o}C.

Specific BACs were chosen from a library of frozen BACs available in the lab which were previously selected for use in a cattle Multiprobe® device from the CH240 BAC library shown in supplementary table 10.1. These were approximately 150 kb in size were selected from the Btau 4.6.1 NCBI database (www.ncbi.nlm.nih.gov) and ordered from the CHORI-240 Bovine BAC library (https://bacpacresources.org) for each autosome and the X chromosome (35).

2.1.2 Qiagen Miniprep kit (Qiagen, Hilden, Germany)

1mL of culture fluid was removed from each falcon tube and was placed into a sterile 2mL Eppendorf tube. BACs were then purified using QIAprep® kit. Bacterial cells were initially resuspended in 250 µl of Buffer P1 containing RNase A, ensuring that no visible cell clumps were present. If LyseBlue reagent was used, it was confirmed to be well-dissolved in Buffer P1. Then, 250 µl of Buffer P2 was added, gently mixed by inverting the tube, and the solution became viscous and slightly clear without exceeding 5 minutes. Next, 350 µl of Buffer N3 was added, mixed thoroughly, and the solution became cloudy. After centrifuging at 13,000 rpm for 10 minutes, a white pellet formed. The supernatant was applied to a QIAprep 2.0 Spin Column, followed by

washing with Buffer PB and Buffer PE. Finally, DNA was eluted by adding 50 µl of Buffer EB or water to the column and centrifuging for 1 minute.

2.1.3 WGA Genomiphi V.2 WGA kit (Cytiva, Marlborough, MA, United States)

GenomiPhi V.2 Sample buffer and Reaction buffer were thawed and alongside DNA samples, were pulsed (up to 6 rpm) on a benchtop centrifuge. 3 µL of each DNA sample were transferred to labelled 0.2 mL tubes with 27 µL Sample Buffer before subsequent mixing and pulse centrifuging. Samples were incubated at 95°C for 3 minutes on PCR block and then placed on ice. An enzyme/Reaction buffer was mixed with the samples. 30 µL of this mix was added to the cooled probe DNA and was pulse centrifuged. Samples were incubated at 30°C for 1.5 hours. Enzymes were inactivated at 65°C for 10 minutes and then put on ice. 60 µL of MBG H20 was added and the solutions were, mixed and transferred to fresh 1.5 mL tubes. 12 µL of sodium acetate/EDTA buffer (mix 50 mL of 3M Sodium acetate (pH 8) and 50 mL of 0.5 M EDTA (pH 8)) was then added. 300 µL of 100% ethanol was added and and mixed gently by inversion and centrifugation for 15 minutes at 11,000 RPM. Supernatant was discarded and 500 µL of 70% ethanol was added and the solutions were centrifuged at 11,000 RPM for 2 minutes. The supernatant was disposed of and the pellet was pulsed in the centrifuge and any significant remaining ethanol was removed and residual ethanol was left to evaporate. 60 µL of 10 mM Tris-HCl buffer (pH 8.00) was added to resuspend.

2.1.4 Nick translation (restriction digestion and fluorescent labelling of cattle BACs)

The DNA concentration of the BAC samples was measured using a nanodrop. 12µL of the solution was then diluted to give a DNA concentration of 166ng/µL using Tris-HCL buffer.

12µL of the diluted solution was then added to a 1.5ml eppendorf with 10xNT buffer, 10xDTT, NucMixA, DNA polymerase, DNAse I molecular biology grade water (MBG H_2O) and 1.5µL of a selected fluorescent label. These labels were a choice of either green Fluorescein isothiocyanate (FITC) (Roche, Basel, Switzerland) or red Texas label (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

The sample was then vortexed and incubated in a PCR Master cycler at 15ºC for 2 hours. This was followed by 10 minutes at 65ºC the samples were then removed from the PCR machine and a gel electrophoresis was performed to see if the BACs in the solution had been digested to a desired length.

If the BACs were not at the desired length 5µL of DNAse was added again to the solution and the solution was put back into the PCR machine. The time the solution would spend in the 15ºC cycle would depend on the lengths of the BACs shown on the last gel produced. This process is repeated until the BACs are within a bracket of desired lengths shown on the gel.

2.1.5 Probe purification

Probes were purified using QIAquick Nucleotide Removal Kit: the probe solution remaining from nick translation was diluted 1:10 with Buffer PNI. This was added to a spin column and was centrifuged at 6,000 RPM for 1 minute. Follow through was discarded. The column was then washed with 750 µl of PE Buffer and centrifuged again at 6,000 RPM for 1 minute. Follow through was discarded. The column was then span for a further 1 minute at 13,000 RPM and follow through discarded. MBG H_2O

was then added to the column and was left to sit for 5 minutes. This was then once again centrifuged at 13,000 RPM for 1 minute. This follow-though contained the purified probes and the spin column was discarded. Purified probes were stored at 4ºC ready for use in fluorescent *in situ* hybridisation.

2.1.6 Multiprobe Device production

The final stage of creating a multiprobe device is to pipette selected probe solutions onto glass apparatus which is made to sit directly upon a template slide as shown in figure 2.1. This glass apparatus is segmented into many boxes, each will have two probes pipetted unto it. 1 FITC labelled probe and 1 Texas labelled probe. Prior to insertion of probes onto the device a master mix is made for each box of the device containing the two probe solutions, hybridisation solution and MBG H_2O. Both probes in each box will bind to a specific chromosome in the target, specifically in the two subtelomeric regions either side of the chromosome.

The number of master mix solutions needed is dependent on the number of chromosomes the target species has. In pigs which have 19 pairs of homologous chromosomes a device will have 19 boxes and thus 19 master mixes need to be produced. The cattle device cannot fit onto 1 glass apparatus so two separate parts are needed for a complete device.

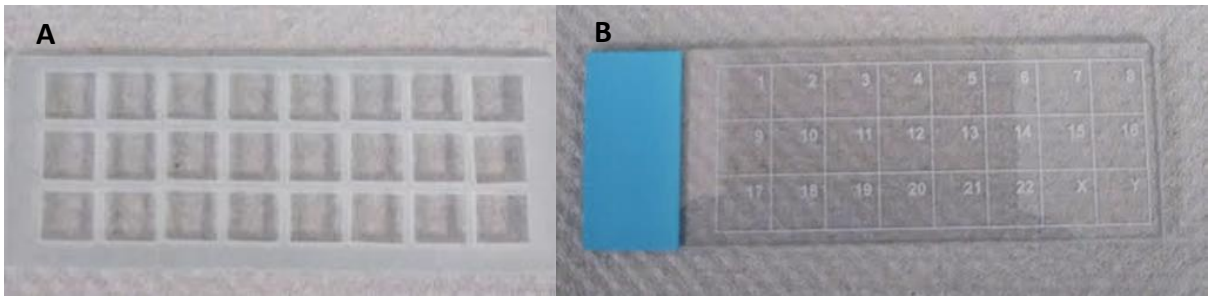


Figure 2.1: (A) An image of the glass apparatus used to make Multiprobe® Devices. Each box will have a mixture containing two probes inserted which will hybridise to a target chromosome. The glass apparatus is used for porcine device as well as the first 24 chromosomes of the cattle genome. A second device with 8 boxes is used for the remaining chromosomes. (B) An image of a template slide. Each box is numbered which corresponds to the chromosome in which probes should hybridise to within this box.

2.2: Fluorescent *in situ* Hybridisation using a premade Multiprobe® device

2.2.1 Mammalian blood suspension culture and harvesting

Porcine and bovine blood samples are received by GriffinLabs. 0.5mL of each blood sample is cultured with 9.5mL PB-Max® Karyotyping medium in a T25 flask. This is incubated for 3 days at 37^{o}C.

Prior to harvesting, 100uL Colcemid (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) is added to the T25 flasks for 30 minutes at 37^{o}C. Colcemid is a mitotic inhibitor which prevents cells in the blood culture specifically the leukocytes from progressing past the metaphase stage of mitosis. The volume of colecemid used at 30 minutes allows for the maximum possible number of cells to progress into metaphase while also maintaining optimal quality of chromosome for microscopic visualisation. When colcemid is left longer than 30 minutes in blood culture a higher number of cells may progess into the metaphase stage however, at this point within many metaphases already present, chromosomes will begin to shorten to a point which makes the chromosomes difficult to visualise under a microscope.

Solutions are transferred to 15ml flacon tubes. Solutions are then centrifuged 1900rpm for 5 minutes to separate cells from the growth medium. The supernatant is removed, 75mM KCl solution is added, and the tubes are incubated at 37^{o}C for 12 minutes. 3:1 methanol: acetic acid fixative is then added to the tubes. The samples are once again centrifuged. The supernatant is removed, and new fixative is added. The sample is again centrifuged, and fixative added, and this process is repeated until the solution appears fully cleaned. The fixed samples are stored in the freezer until FISH.

2.2.2 Slide preparation

Template slides with a number of boxes equal to the number of chromosomes in a target species are used. Approximately 1.5ul of metaphase suspension was pipetted in each box. The slides were then put through a 5 second 70% acetic acid wash. This followed by a dehydration series as follows: 2 minutes in 2x saline sodium citrate (2xSSC), 70%, 85% an 100% ethanol at room temperature. Slides are then dried before being placed face up on a 37ºC hotplate.

2.2.3 First-day FISH

1uL of hybridisation solution is pipette directly onto each box of the Multiprobe® device. This device is than place onto the sample slide so that each box on the sample slide matches each corresponding box found on the wet Multiprobe® device. Slides are left on the 37ºC hotplate for 10 minutes before being transferred to a 75ºC hybrite for 5 minutes. Sample slides are then incubated with the device still mounted in a hybridisation chamber placed in a water bath at 37ºC overnight.

2.2.4 Second-day FISH (16-24hrs later)

Template slides are removed from the 37ºC water bath. Devices are removed from the slides. The slides are then immersed for 2 minutes in 0.4XSSC at 72ºC followed by a 30 second immersion in 2xSSC with 0.005 Tween-20 at room temperature. This removes excess unhybridized probes from the slide. On a coverslip a couple drops of DAPI in VECTASHIELD® antifade medium is added. This coverslip is placed face down on the wet slides immediately proceeded SSC immersion.

DAPI is a fluorescent stain which binds strongly to DNA thus making the chromosomes of the target species nuclei visible under a fluorescent microscope.

2.2.5 Fluorescent Microscopy of a sample slide with hybridised FISH probes

Slides were kept stored in covered slide folders in a lab refrigerator ~4^{o}C. Fluorescent microscopes were situated within dark rooms to prevent the signal of the fluorescent probes being dimmed through its reaction with ambient light. Fluorescent microscopes used also were equipped with 3 light filters one for each fluorescent probes as well as a filter for DAPI. Each chromosome box (figures 12B) was scanned for metaphases, which are recognised as a circular bundle of visible chromosomes. Once suitable metaphases were found the metaphase was zoomed in on and the fluorescent green and red filters were adjusted so that signals double signals on two different chromosomes for each filter were visible. Images were taken. If any probes were found on an unexpected chromosomes 3-5 more metaphases were then screened and imaged to confirm or disconfirm a diagnosis. Image capturing was performed using an Olympus BX61 epifluorescence microscope with a cooled CCD camera and SmartCapture (Digital Scientific UK) system.

3: Results for Specific Aim 1

3.1: Specific aim 1a: Screening pig blood samples using a Fluorescent *in situ* Hybridisation Multiprobe® Device.

3.1.1 Background

The first specific use of FISH this thesis will focus on, is as a screening tool for porcine samples which involved blood samples being delivered our FISH specialising lab, leukocytes being cultured and harvested, the mounting of these cells onto slides and the subsequent application of a FISH Multiprobe® device. The outcome of this specific method is the attachment of fluorescent probes to the sub-telomeric regions of each chromosome in animals diploid set. This allows us to check metaphases and individual chromosomes for abnormalities with RTs being by the far most common abnormality in porcine samples or Robertsonian translocations (RobT) being the most common in cattle. For example, in a case where a reciprocal translocation has occurred, one of the probes targeting a specific chromosome (e.g. chromosome 3) will be present on

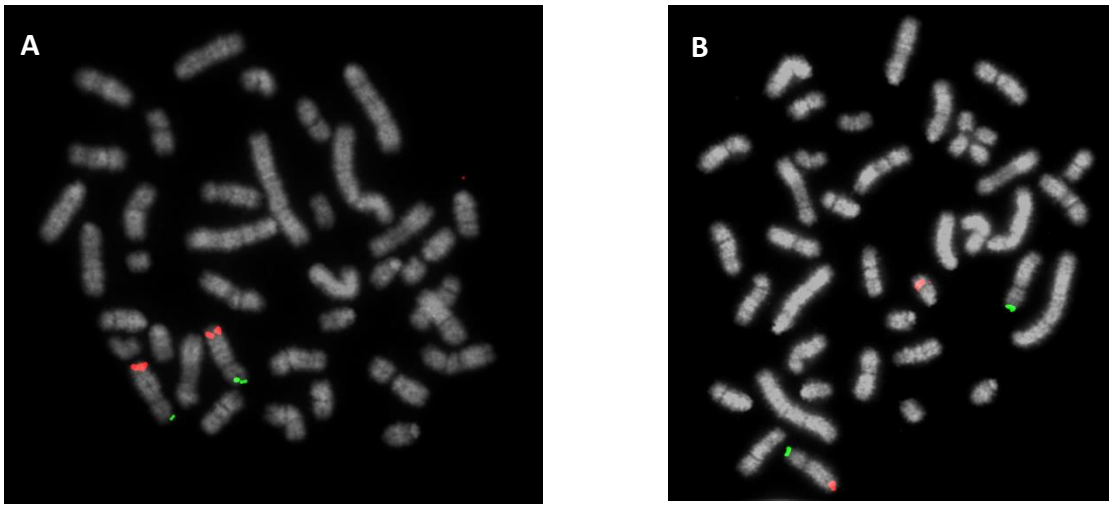


Figure 3.1: Example of a reciprocal translocation in *Sus domesticus*. Both of these images show metaphases where probes are targeting chromosome 3. A) This metaphase is of a normal individual, therefore 2 sets of probes appear either side of each homologous chromosome 3. B) This metaphase is of an individual with a reciprocal translocation. The Texas Red-based probes from one of the larger chromosomes appears on a different chromosome (chr17).

one of the chromosome in which the part of the chromosome has been exchanged with (e.g. chromosome 17) (Figure 3.1).

As the probes are in the sub-telomeric regions, and reciprocal translocations involved an exchange of DNA between two different chromosomes, the probe targeting the second chromosome in which the other probe has moved (chr 17) will also be visible on the original chromosome (chr3). The benefit of using FISH over standard karyotyping in this regard is that a standard karyotype fails to accurately detect reciprocal translocations under 2-3Mb in length whereas FISH is able to detect these more subtle cryptic translocations.

Aneuploidy is also easier to locate due to the presence of an abnormal number of chromosomes with probes attached within a specific box. Mosaicism/chimerism is visualised by the presence of a differing number of a particular chromosome in 2 separate cells. This is easily visible with FISH. Screening cattle for RobTs can be achieved with karyotyping however using FISH offers a faster approach which is less prone to error in determining the exact chromosomes effected as specific chromosomes are labelled in separate regions of the slide.

The purpose of the first study is to evaluate recent data collated from a porcine chromosomal aberration screening service ran by GriffinLabs at the University of Kent. The results cover the period throughout 2021 and until May 2022. The following data and images were collected throughout the mentioned period. Where possible a basic unbanded karyotype was performed in the below data to test whether karyotypes could have potentially be undertaken and if they would have been as sensitive as FISH in locating translocations.

724 boars and 51 sows were screened for chromosomal abnormalities using Multiprobe® FISH devices. Out of these 14.5% of pigs presented with a chromosomal abnormality. Reciprocal translocations were by far the most common aberration found making up 13.9% of the boars screened. There were also 2 chimeric XX/XY boars found in this time period and 2 found with complex translocations (T). The estimated incidence of *de novo* reciprocal translocations based on this data was 0.77%.

Table 3.1: Summary of pigs screened using Fluorescent in situ hybridisation with a Multiprobe® device from January 2021 until May 2022. Table shows incidence of reciprocal translocation (RT), complex translocations (T), XX/XY chimeric and normal individuals.

Abnormality	**Carriers**	**Percentage(%)**
RT	108	13.9%
T	2	0.3%
XX/XY Chimeric	2	0.3%
Normal	663	85.5%
Total	775	100.0%

3.1.2 Reciprocal translocations

Of the 108 reciprocal translocation carriers, 100 were RT(1;2) carriers almost all of which were offspring of 1 or 2 individuals. 4 were RT(9:18) carriers from the same batch indicating the translocation had also been passed onto offspring. and the rest were *de novo* RTs (meaning they occurred only in that individual) which occurred independently and likely had severe consequences to the individuals fertility or these pigs were not used for mating as no other individuals (i.e. offspring) were found with these translocations.

Table 3.2: Incidence of specific reciprocal translocations (RT) found in pig samples screened for chromosomal abnormalities using Fluorescent *in situ* Hybridisation with a porcine Multiprobe® device between January 2021 until May 2022.

Type of RT	Number of Pigs with RT	Percentage(%) of Total RTs
1.2	100	92.6%
9.18	4	3.7%
1.3	1	0.9%
9.10	1	0.9%
10.17	1	0.9%
7.17	1	0.9%

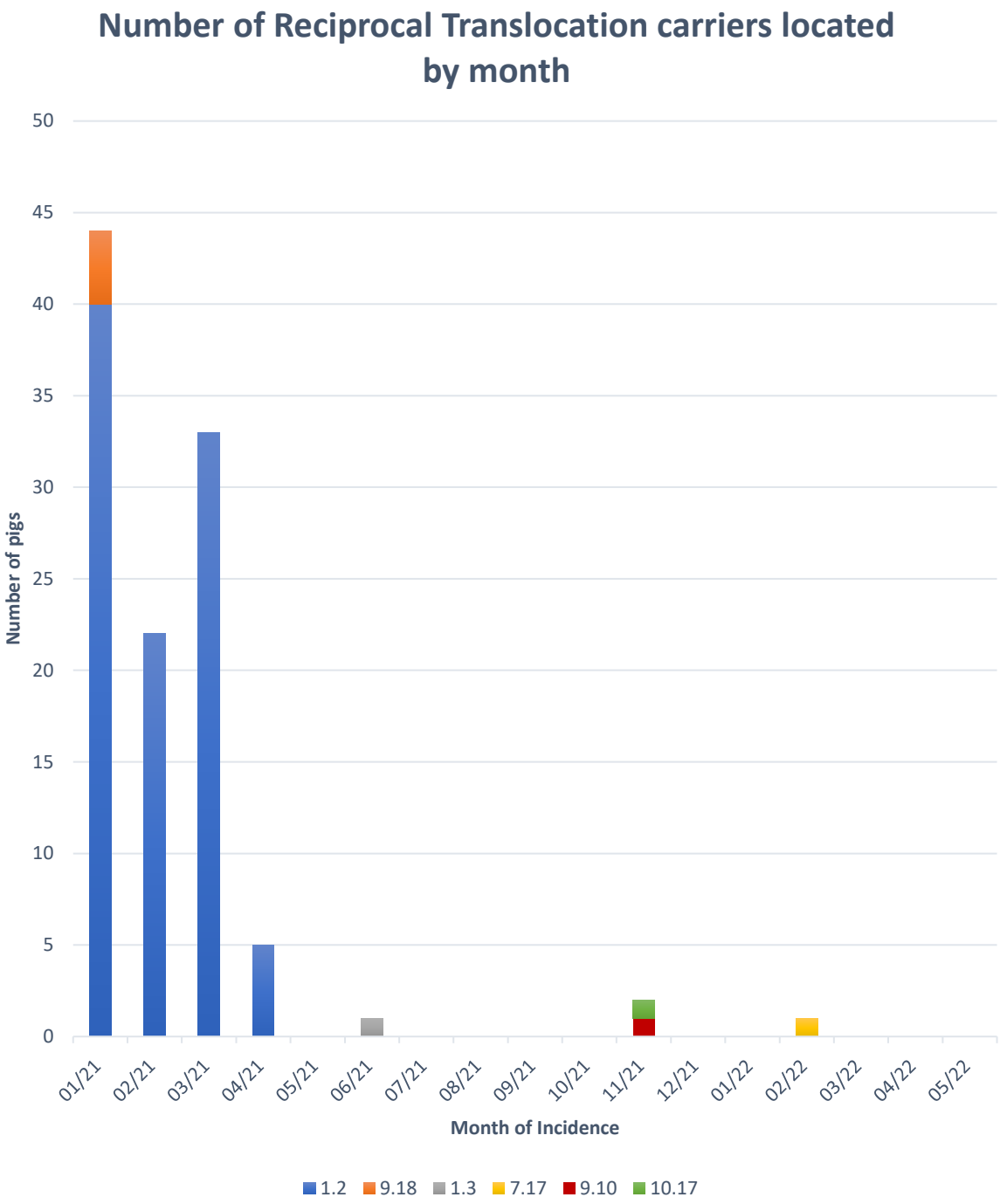


Figure 3.2: By month number of pigs diagnosed with specific reciprocal translocations (reciprocal translocation colour key at bottom of figure) found between the periods of January 2021 until May 2022 while performing chromosomal abnormality screening using fluorescent in situ hybridisation.

RT(1;2)

These results display an extremely high frequency of RT(1;2) shown in figure 3.3. This translocation likely occurred *de novo* during restrictions in place in 2020 because of the COVID-19 pandemic. This allowed the translocations especially RT(1;2) to spread for a prolonged period of time through the breeding population. This client reported greatly reduced litter sizes and reduced fertility in a large number of pigs towards the end of 2020 and beginning of 2021 and therefore send a large number of potential carrier pig blood samples to GriffinLabs. RT(1;2) individuals were continuously discovered from 01/2021 until 04/2021. These individuals were removed from the breeding population as there was a notable reduction in cases in 04/2021 and all RT(1;2) were removed from the population by 05/2021 despite continued reception of samples from this population.

A subsequent karyotype of this translocation shown in figure 3.4 demonstrated that there was an unnoticeable morphological difference when observed in a fluorescent microscope image between the translocated chromosomes and the normal homologous chromosome meaning karyotyping alone would have likely been insufficient in detection.

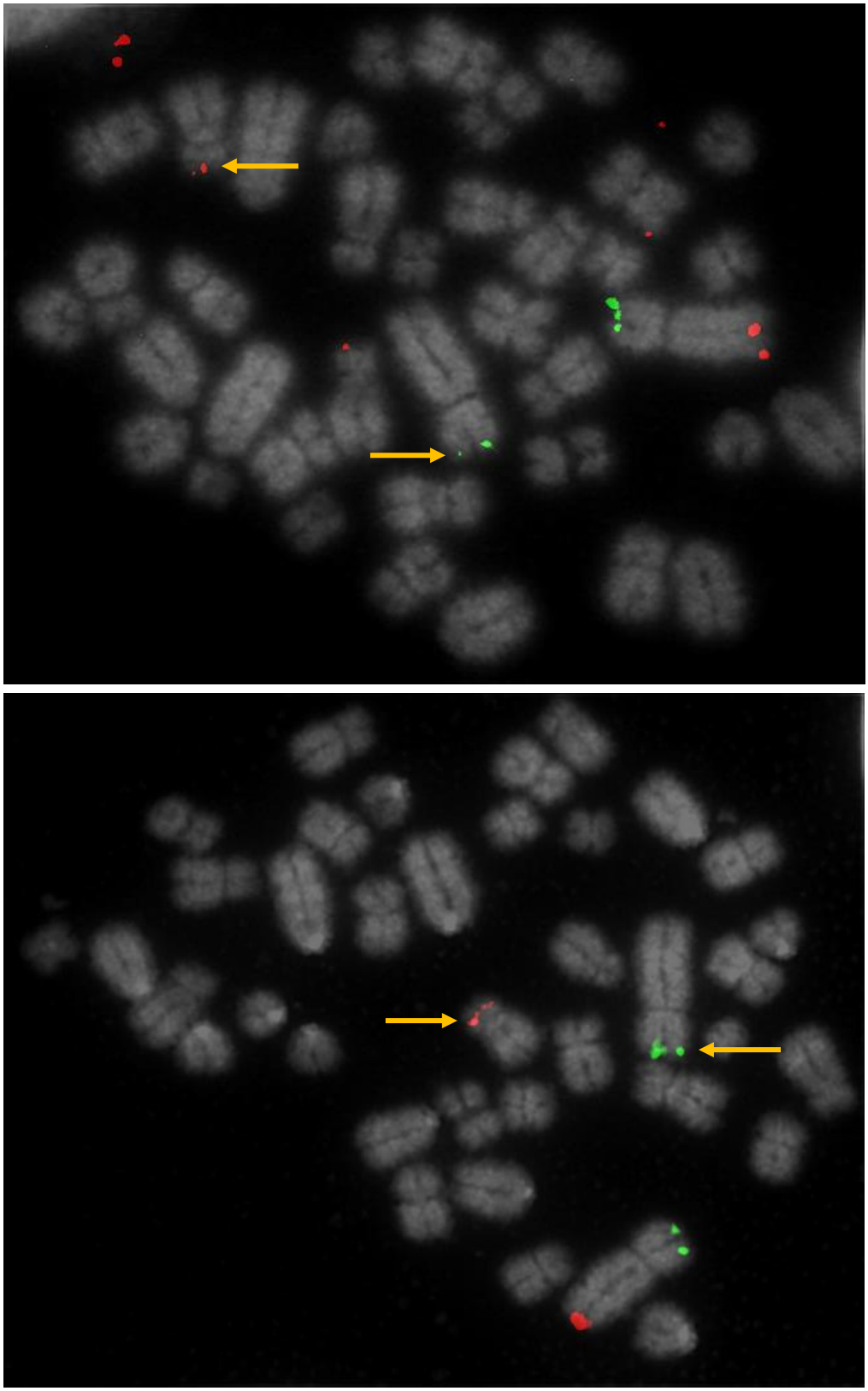


Figure 3.3: Images of 2 of the cases which lead to diagnosis of a cattle with Reciprocal translocation(1;2). Both images are of metaphases which have had porcine chromosome 1 targeting probes applied. The arrows point to normal FITC (green) signals hybridised to the correct chromosome 1 and the Texas (red) probes attached to the incorrect chromosome 2 thus leading to diagnosis of RT(1;2).

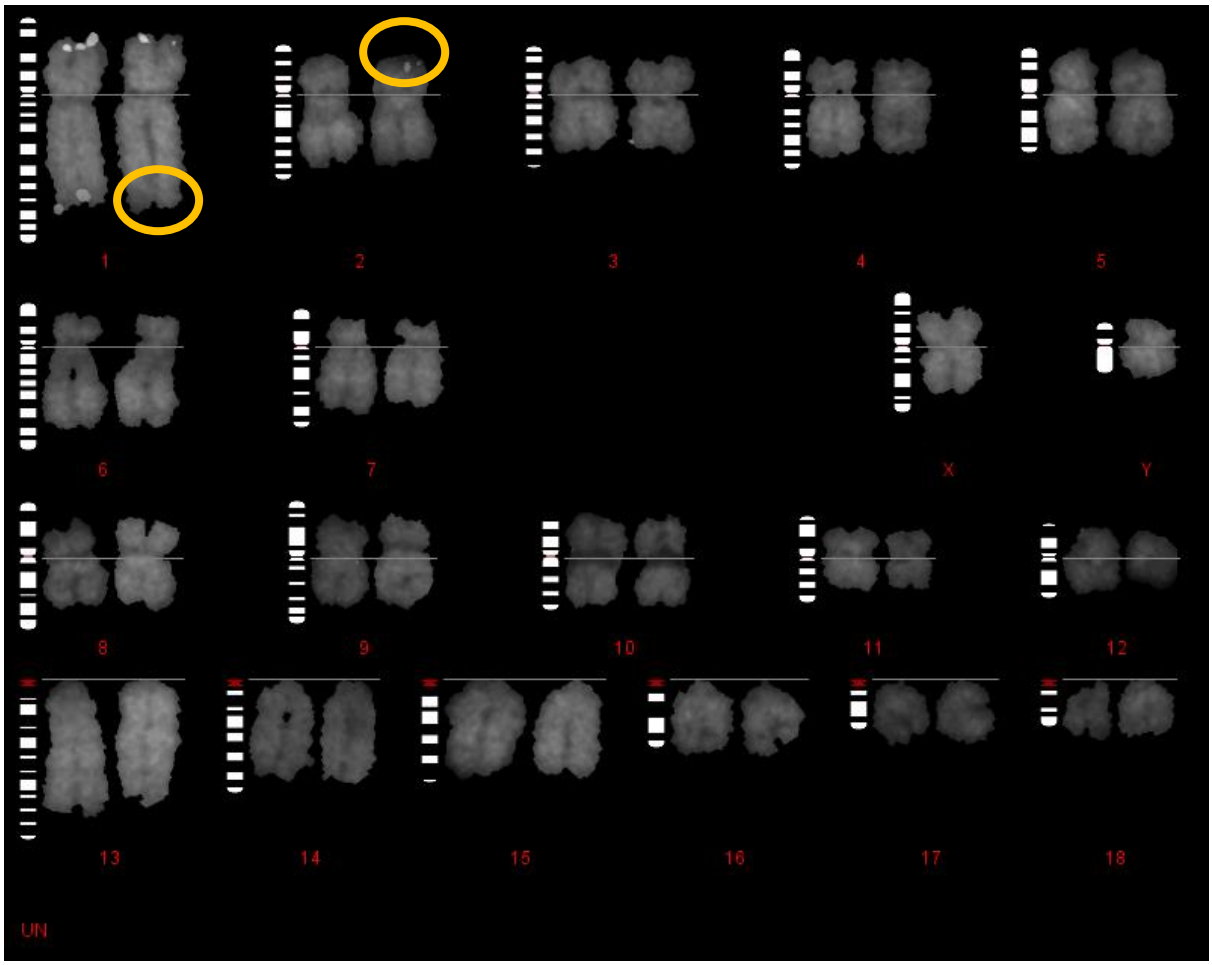


Figure 3.4: Unbanded karyotype image using the image on figure 3.3 of a porcine metaphase presenting with Reciprocal translocation(1;2). Inaccuracies in the order of the karyotype are likely due to difficulties in differentiation due to no banding being used. However, as signals can be seen above in greyscale chromosomes 1 and 2 it is certain that these chromosomes are in the right place.

RT(9;18)

Another translocation which was located within the same group of breeding boars was the RT(9;18) translocation (figure 3.5). This translocation had far fewer carriers however as mulitple members from the same mating group had been found it is likely this translocation had been passed down from one parent where the translocation occurred *de novo* and several offspring had inherited this translocation heterozygous. This translocation was located before significant spread in the breeding population. Curiously, all four of the RT(9;18) translocations discovered in 2021 were all found in the same batch (PEU0121D). Moreover, this batch also contained 16 individuals who were carriers for the RT(1:2) translocation which were likely to be in the same breeding population however no individual had both translocations present.

A karyotype to determine whether this translocation could be found with karyotyping alone could not be undertaken due to poor quality in samples due to delayed delivery times of blood samples resulting from the Covid-19 pandemic. Despite difficulties in differentiating chromosomes, FISH probes for particular chromosomes are segregated. This meant FISH analysis could still be undertaken and the translocation should be present on the corresponding two separate boxes on the microscope slide.

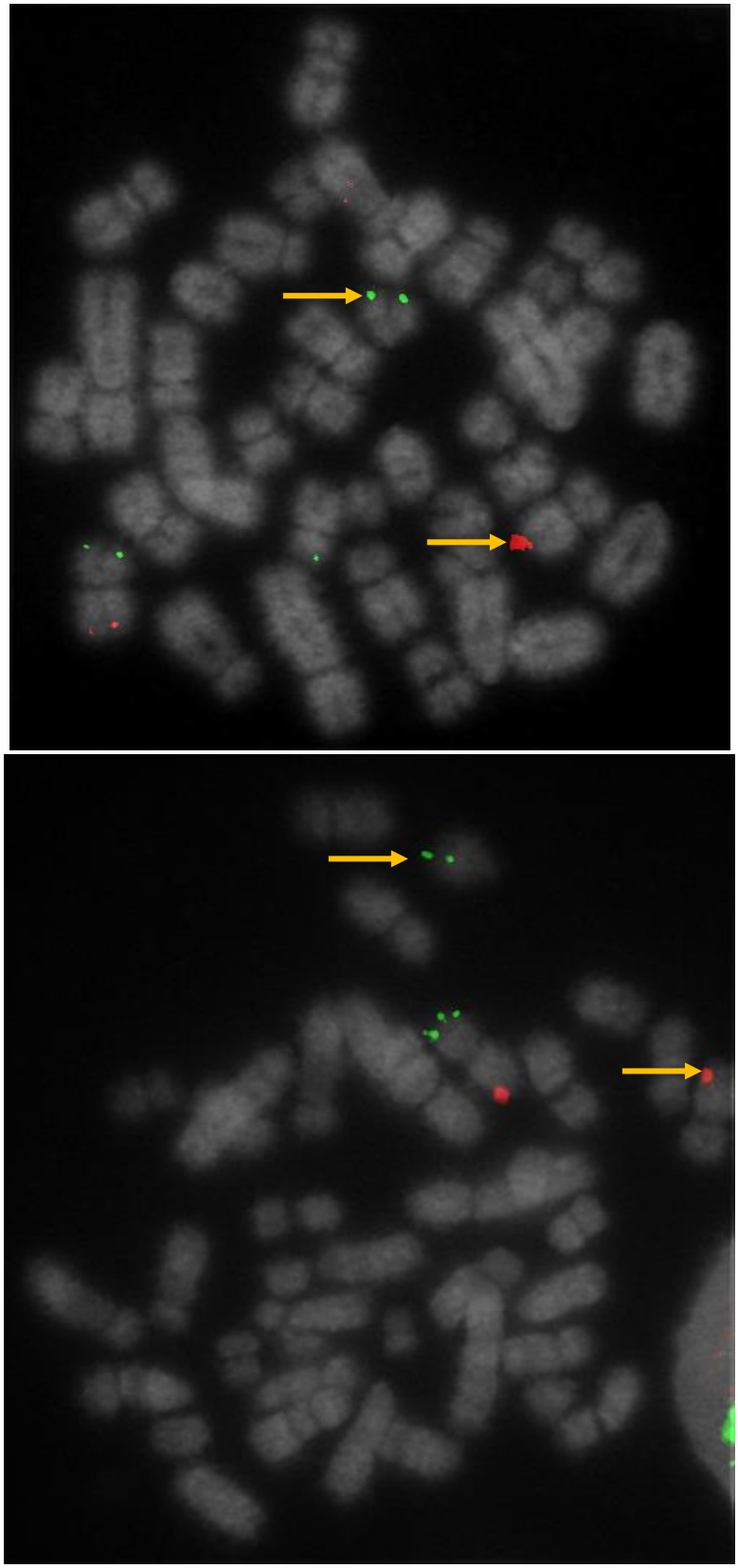


Figure 3.5: Images of two different reciprocal translocation(9;18) diagnosed boar samples. Both metaphases have chromosome 9 targeting probes applied. The FITC (Green) probes are seen on the smaller chromosome 18 therefore part of the p-arm of chromosome 9 has translocated with chromosome 18 while the Texas (Red) probes remain on chromosome 9.

RT(1;3)

The reciprocal translocation RT(1;3) imaged in figure 3.6 was also found *de novo* in a single isolated case. This translocation appeared far larger than the previous two and shows up very clearly even on a karyotype which is shown in figure 3.7. The translocation involves a large portion of the q arms of chromosome 1 being translocated with a far smaller portion of the p arm of chromosome 3. This creates a very clear extension of chromosome 3 and shortening of chromosome 1. This translocation has previously been reported in several previous reports (60). Information was not provided to whether this boar had been used for breeding before we received this sample however this is unlikely, and no other boars were found with this translocation.

A karyotype was also made which shows significant changes with chromosome 1 and 3. Low quality metaphase image which may be down to a poor sample does make the chromosomes in the karyotype fuzzy and lacking in detail (figure 3.7). However, as there is a large enough change to chromosome 1 it is clear that a karyotype would suffice in diagnosing this translocation.

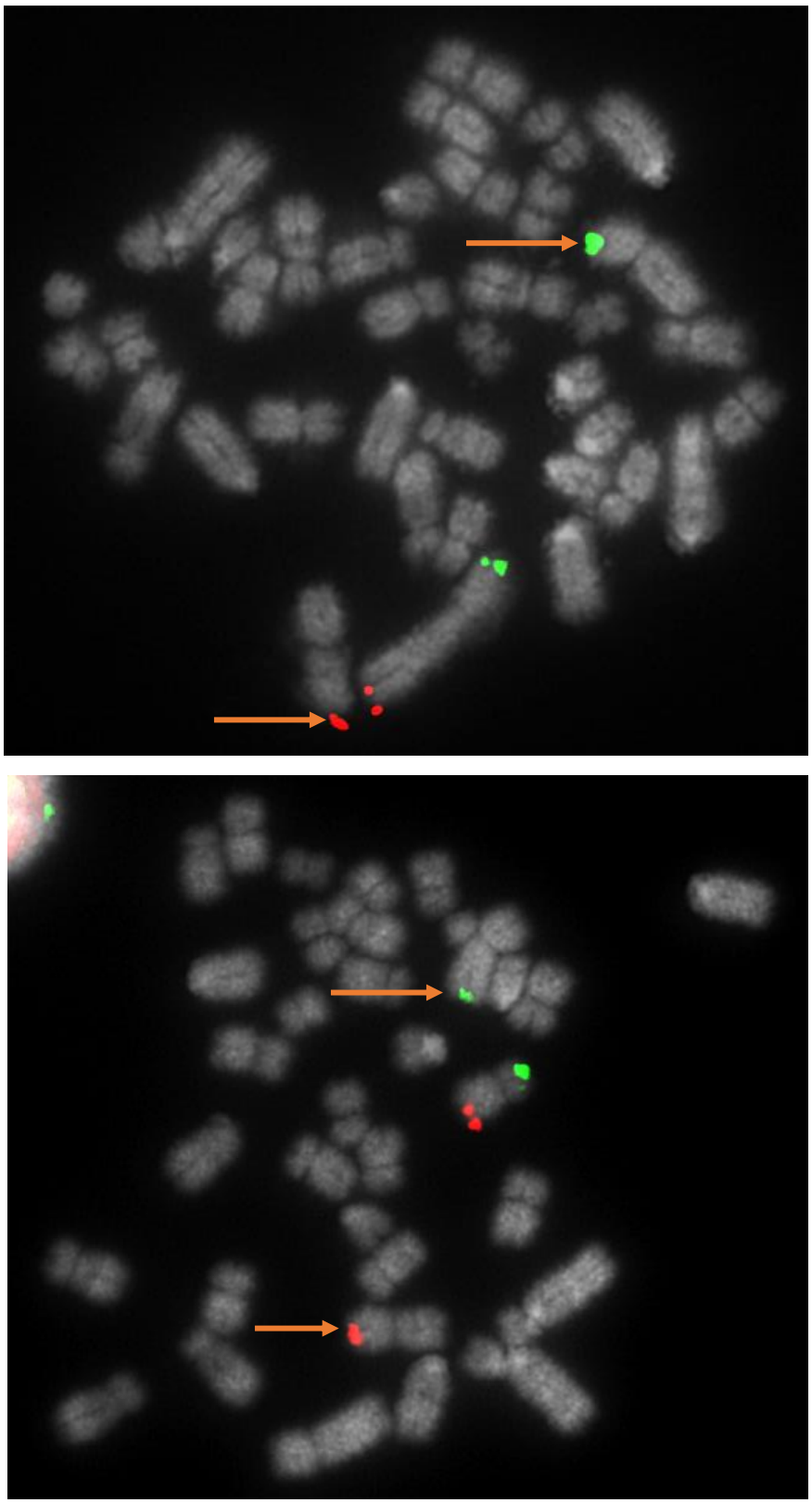


Figure 3.6: Images of two metaphases from a reciprocal translocation(1;3) diagnosed boar. The top image is a metaphase with chromosome 1 targeting probes applied and the bottom is a metaphase with chromosome 3 targeting probes applied.

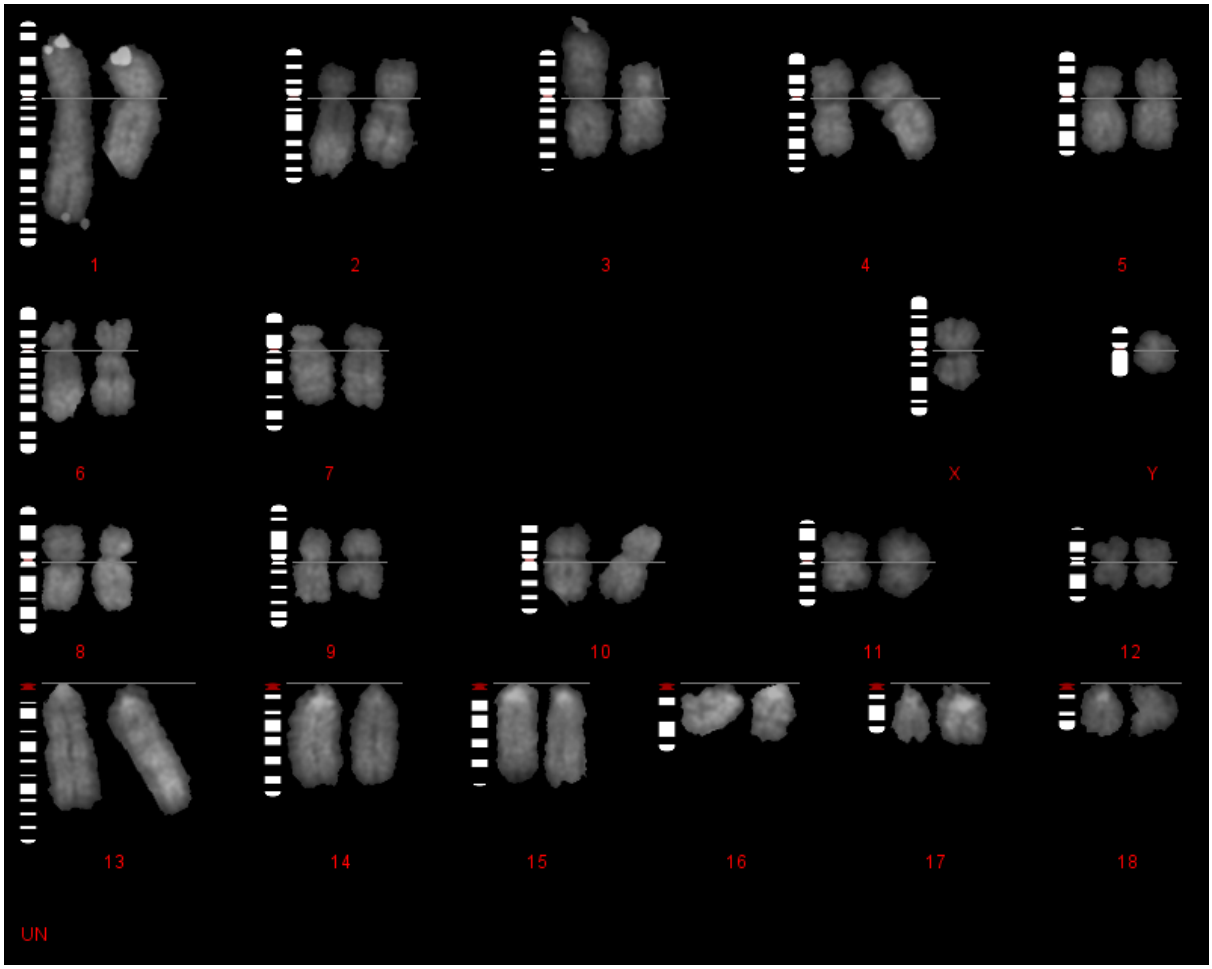


Figure 3.7: Unbanded karyotype of reciprocal translocation(1;3) diagnosed boar using the above image of a metaphase with probes specifically binding to chromosome 1.

RT(9;10)

The translocation RT(9,10) was located in November 2021. Once again this was an isolated case 18 tested male pigs within this batch. As no other individuals displayed this translocation it is likely this translocation was *de novo* and its discovery prevented it being passed on to any other breeding pigs. This translocation is rare however has previously been reported in 2021 in Australian swine herds (61). Once again due to a poor-quality sample a sufficient karyotype could not be made to see if this translocation could be found without using FISH. In the abovementioned study a karyotype was made and was high enough quality to determine that a karyotype was sufficient for diagnosis of this translocation. However, translocations can in different breakpoint locations within a chromosome therefore this translocation may not be the exact same one reported in the Australian study. FISH was however sufficient to make a diagnosis in this case. Images of this translocation shown in figure 3.8 are in greyscale due to temporary fault in microscope camera. This meant that 2 images were taken of the same metaphase shown below to distinguish between the two signals clearly.

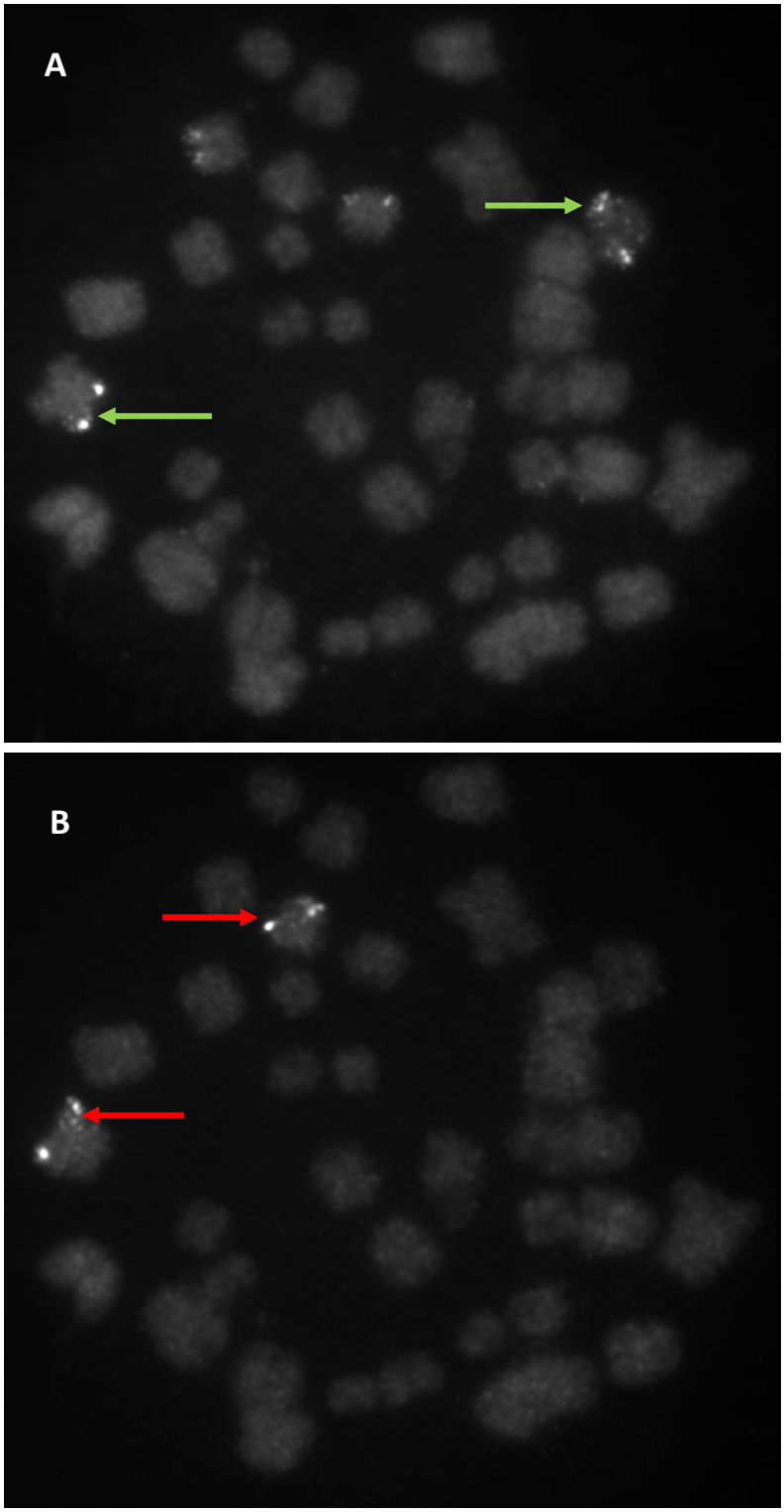


Figure 3.8: Image shows a boar presenting with reciprocal translocation(9;10). A)The positions of FITC (green) p-arm probes as shown by arrows targeting chromosome 9. FITC signals are both on chromosome 9. Faint off-target signals as seen above are common in FITC probe targeting chromosome 9. B) Image shows strong Texas signals in chromosome 9 (bottom left) and chromosome 10 (top right).

RT(7;17)

Translocation (7,17) was the most recently discovered *de novo* translocation found. This was located as an isolated case within a batch of 6 samples. Translocations between these chromosomes have previously been discovered in 1982 in Sweden(62).

RT(10;17)

Reciprocal translocation (10;17) was once again an isolated *de novo* case which was likely not spread to other individuals in the breeding group. This translocation was found in November 2021 the images of which are shown in figure 3.9 and was found in an otherwise chromosomally normal group of boars.

The images taken were not suitable for use in a karyotype however, FISH images taken show clearly that both in metaphases with chromosome 10 targeting probes and metaphases with chromosome 17 targeting probes, that the probes to one end of a single chromosome have instead targeted a different chromosome indicating a translocation. The RT(10,17) translocation had been previously reported in 2007 in a French study which only located RTs by way of karyotype suggesting that the translocation could be large enough to detect purely by karyotyping(63).

Figure 3.9 shows two metaphase images of one reciprocal translocation(10;17) diagnosed boar. Part A is of a metaphase that has chromosome 10 targeting probes applied. As can be seen by the normal chromosome being considerably larger and metacentric compared to the small acrocentric chromosome (chr17) where the probes have been translocated to. Translocated chromosomes are highlighted by orange arrows.

Part B of figure 3.9 is a metaphase of the same individual with chromosome 17 probes applied.

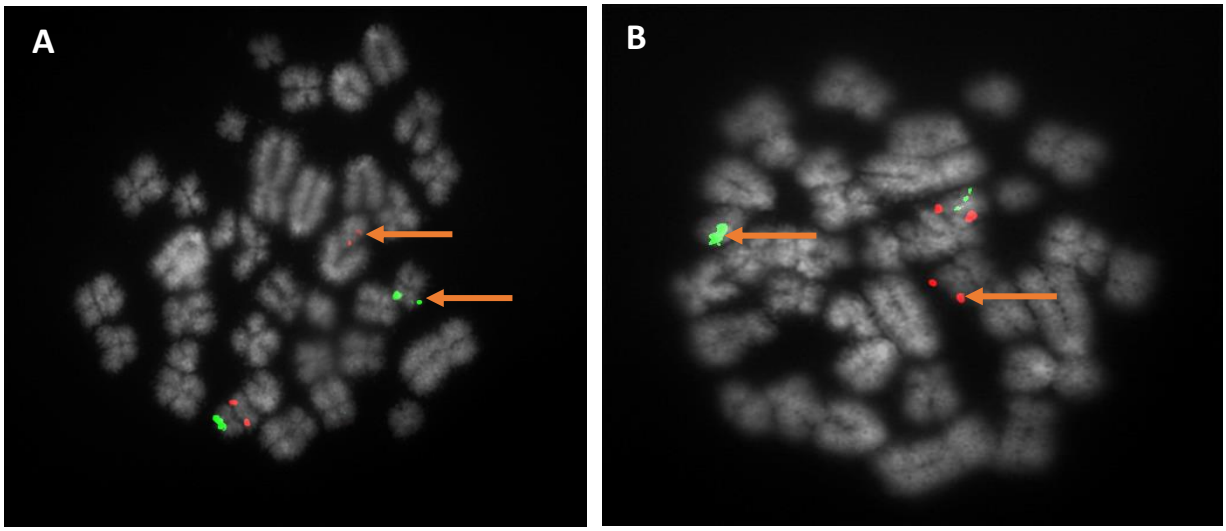


Figure 3.9: Two metaphase images of one reciprocal translocation(10;17) diagnosed boar. A) Image of a metaphase that has chromosome 10 targeting probes applied. As can be seen by the normal chromosome being considerably larger and metacentric compared to the small acrocentric chromosome (chr17) where the probes have been translocated to. Translocated chromosomes are shown orange arrows. B) Image is a metaphase of the same individual with chromosome 17 probes applied.

Chimeric XX/XY

In the period covered in thesis two cases of chimeric XX/XY pigs have been located out of 775. Around this level of prevalence appears consistently be the case in studies collating data from screening pigs for chromosomal aberrations. These aberrations are not common but have widely been reported in many studies (41, 60). In these cases, not only metaphases were checked but a large volume of interphases with some only showing 1 set of FITC and Texas probes and others showing two sets of probes. This indicates some interphases contain two X chromosomes, while others only contain one.

As X probes don't target the Y chromosome in pigs it is feasible to suggest that the individual could also be mosaic XY/XXY which may be missed using only FISH due to the Y chromosome often being hard to differentiate while looking at it in the context of a metaphase. A karyotype may better fully distinguish between these two abnormalities however either abnormality will most likely result in the removal of the boar from the breeding population regardless as both may cause some level of infertility.

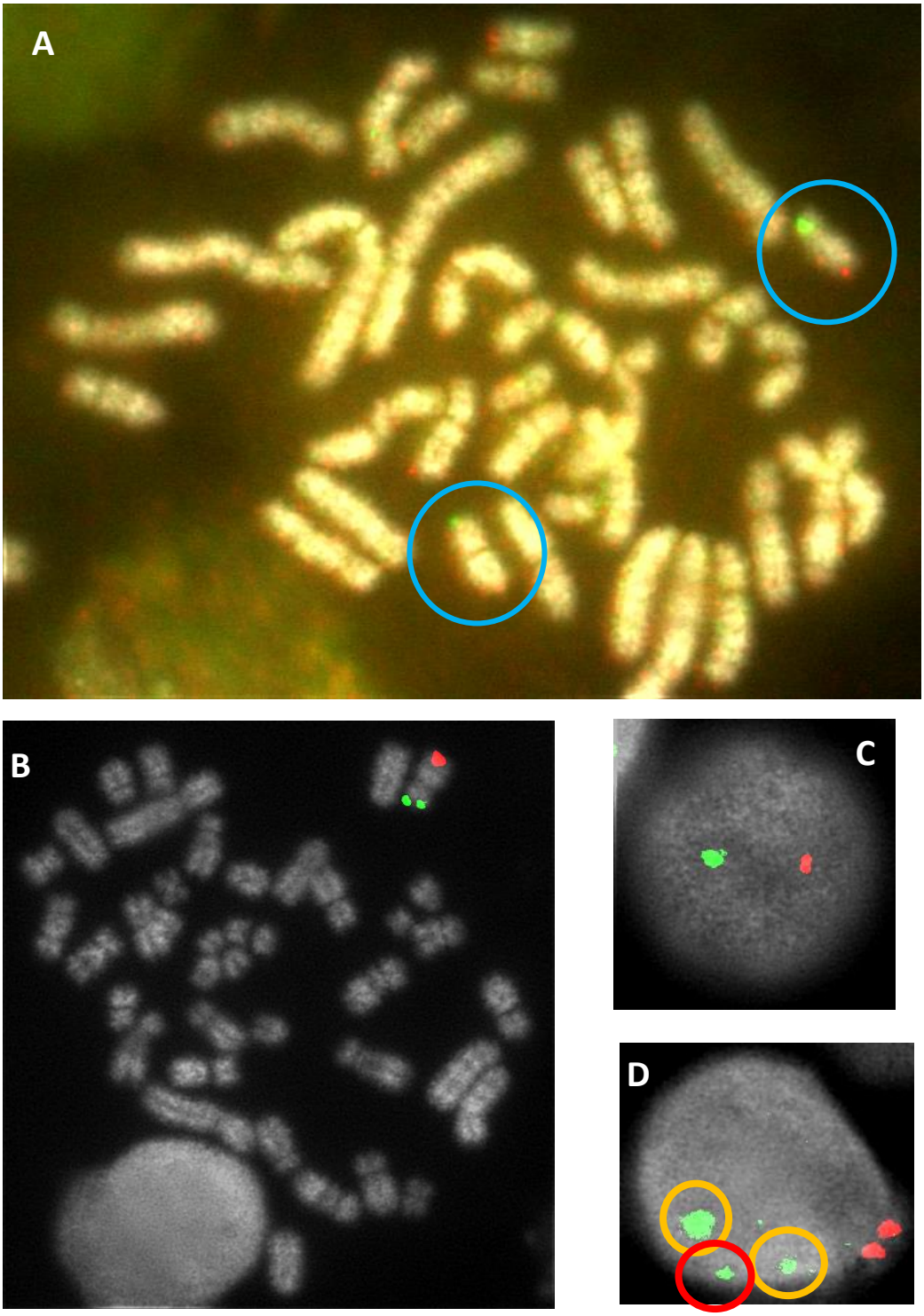


Figure 3.10: Images taken to diagnose a chimeric XX/XY boar. A) Metaphase with X targeting probes applied showing an XX metaphase. B) XY metaphase. C) Interphase showing only one pair of signals which can thus be assumed to be XY. D) XX interphase, the image shows 2 pairs of FITC(green) signals however some off-signalling was present causing the appearance of what looks like another signal. This was clearly not the case when observing under microscope however, as there are 2 clear pairs of Texas (red) signals present the image still indicates an XX interphase.

3.2: Specific Aim 1b: Screening Cattle Blood Samples Using a Novel Bovine Fluorescent *in situ* Hybridisation Multiprobe® Device.

3.2.1 Background

The chromosomal screening service provided was also extended to cattle albeit to a far lesser extent. During the period 2021-May 2022 only 3 bulls were screened for chromosomal aberrations each sample being received individually and from different times and locations/providers from one another (table 3.3). The providers of these cattle samples typically had already predicted or had found the presence of an abnormality and were more interested in an exact diagnosis or confirmation or diagnosis.

Table 3.3: Incidence of abnormalities in cattle cytogenetically screened using a FISH Multiprobe® device approach.

Abnormality	**Carriers**
RobT(1;29)	1
Aneuploid	1
Normal	1

3.2.2 Robertsonian Translocation(1;29)

As previously stated in the introduction the Robertsonian 1;29 translocation is very common in almost all cattle breeds and has a prevalence rate of up to 60% in certain breeds. The particular case imaged in figures 23 and 24 was found in May 2022 and was sent as a single sample. Multiprobe® FISH analysis quickly confirmed a suspected diagnosis of RobT(1;29) (figure 3.11). Karyotyping also offers as effective diagnosis even without banding as the presence of this translocation makes an obvious observable change in the chromosome set of the carrier (figure 3.12). This is because one chromosome will be missing from the karyotype while another will have a significant increase in size and change in centromere position due to the fusion.

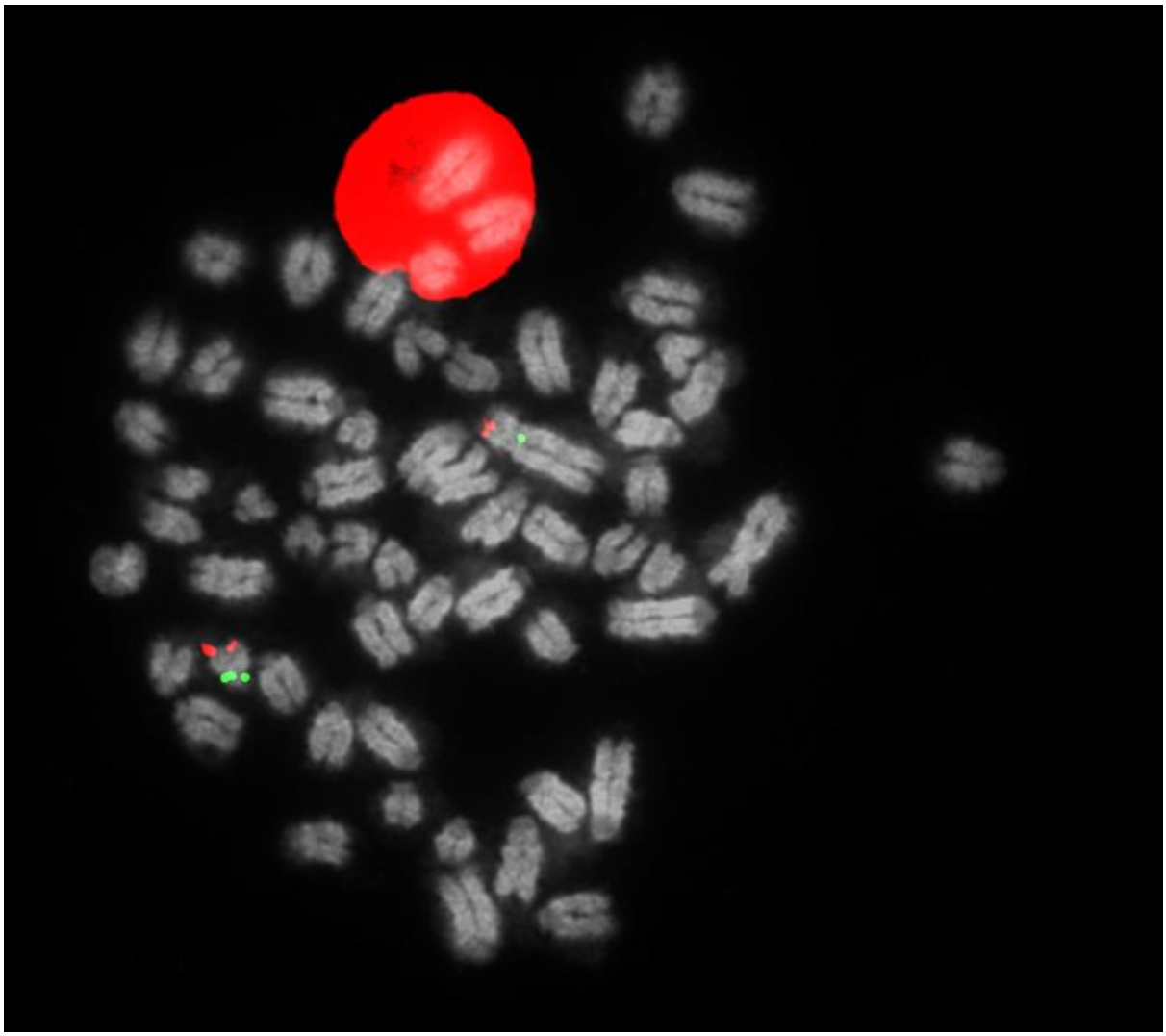


Figure 3.11: Metaphase of RobT(1;29) diagnosed cattle. Probes used targeted cattle chromosome 29. The image shows one of the smaller chromosomes 29's has fused via its centromere into the centromere of a much larger chromosome (chromosome1).

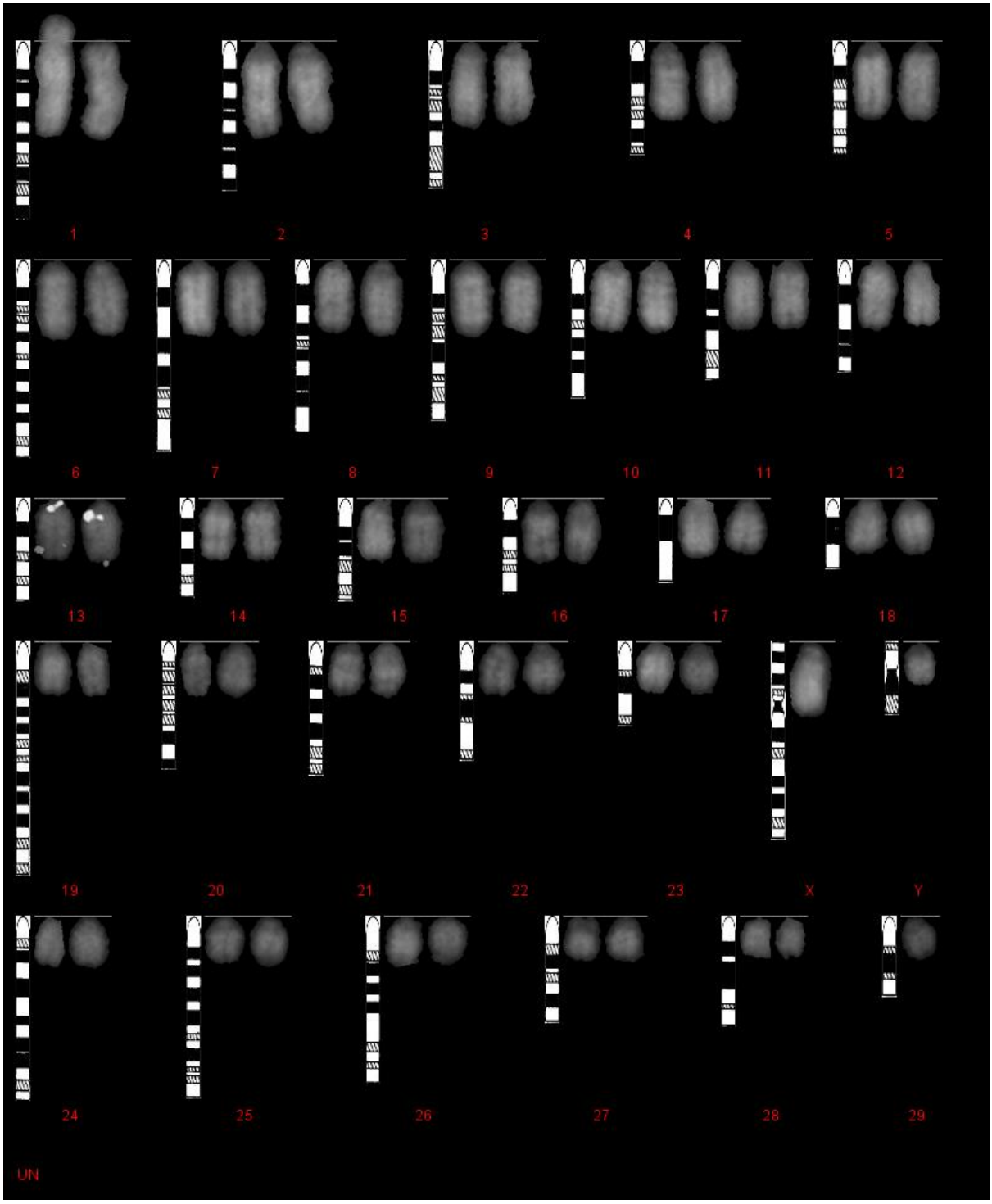


Figure 3.12: Karyotype of the RobT(1;29) diagnosed cattle using figure 3.11 image. Image shows an extension to chromosome 1 beyond the centromere and a missing chromosome 29.

3.2.3 Aneuploidy

An individual cattle sample displaying aneuploidy for the sex chromosomes (XXY) was found as shown in figure 3.13. Only Texas was used in the image taken which more clearly displays 3 chromosomes with signals when there should only 2 as X targeting Texas red-based probes also target Y in cattle chromosomes unlike in pigs where the Y chromosome never binds to any probes.

Aneuploidy can also be easily found using karyotyping due a loss or gain of a whole chromosome is easily observed.

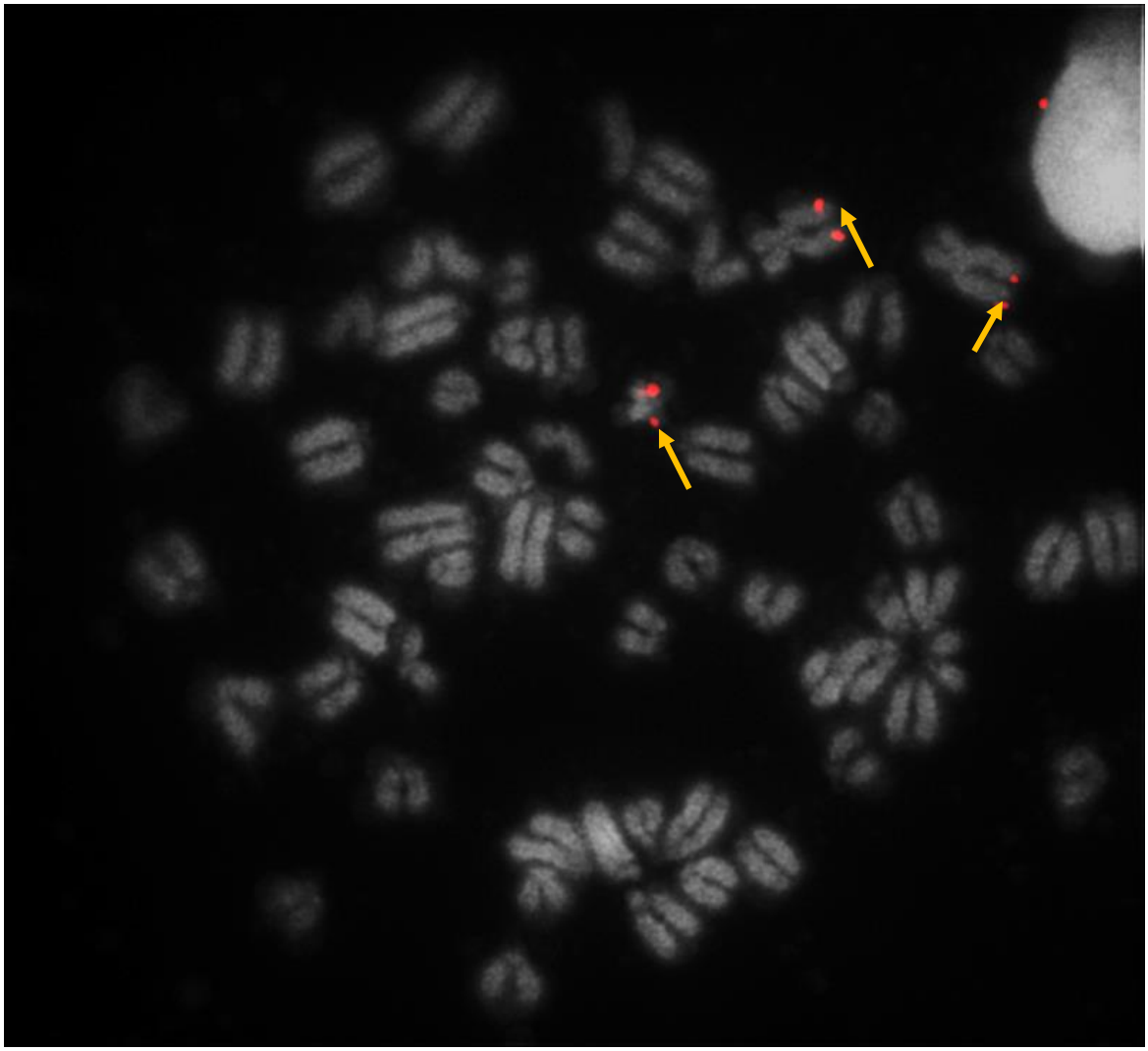


Figure 3.13: Image of metaphase of an aneuploid XXY diagnosed cattle sample. Only Texas (Red) FISH subtelomeric probes were used as the shorter Y chromosome only binds to the X targeting Texas probes.

4: Results for Specific Aim 2: Designing a Novel Clinical FISH Multiprobe Ovine Device

4.1: Background

For the next section of this thesis a cattle device was used on a control slide with a normal mounted cattle sample and a healthy sheep sample. Comparisons in which chromosome and the chromosomal location of the cattle probes on the sheep chromosomes was then made using the cattle control slide as a reference. The study also intended to use a dromedary camel due to the potential of a device as they are also often carefully bred. This was not possible however as the camel samples available failed to show clear metaphases and were unusable. The sheep sample produced chromosomes which were clear enough to distinguish and perform FISH with this said the sample ideally should have been higher quality for more reliable results. Assessments made were purely qualitative and were judged on whether or not a clear signal pair could been seen under microscope in a clear location within a chromsome with little to no off target singalling. Too much signalling would suggest that the given probe did not have strong enough binding affinity for a clinical multiprobe device and another alternative probes should be found.

Sequences of bovine BACs were found and the NCBI Blast tool was used to check for the presence of these sequences in the ovine genome and therefore use this information as a predictor of successful binding of probes on ovine chromosomes. This was largely unsuccessful as these sequences would prodominently only have matches to small constituent segments (e.g. 50bp) relative to the whole sequence of the BAC (~200,000bp). This therefore meant that it was difficult to know how these parts

arranged in the sheeps genome and the percentage of each BACs sequence within the ovine genome.

Due to issues with the longevity of stored probes needed for the cattle device and potential errors which were made evident in the control slide, probes targetting chromosomes 2, 9, 26, 27 and 28 were excluded from the study. The q arm probe (Texas) for cattle chromosome 22 and p arm probe for chromosome 23 were also excluded due to inactivity on the control sample.

Of the remaining probes 38 of 50 cattle probes clearly attached to a clear location of the chromosomes in sheep metaphases giving an overall hybridisation success rate of 76% in this specific sheep sample (table 4.1 and 4.2). 5 of 24 viable FITC green-based probes did not hybridise and 5 of 25 Texas Red probes did not successfully hybridise.

Table 4.1: Summary of hybridisation success rates of cattle subtelomeric FISH probes on sheep chromosomes

Worked?	P-arm (FITC/Green)	Q-arm (Texas/Red)
No	5	5
Yes	19	19
Total	24	24

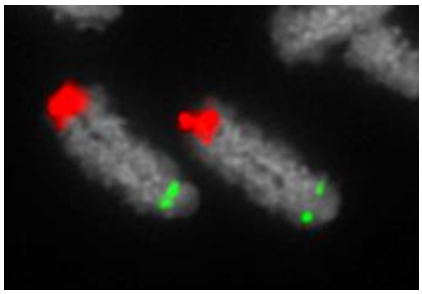
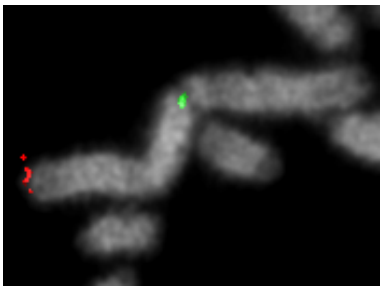
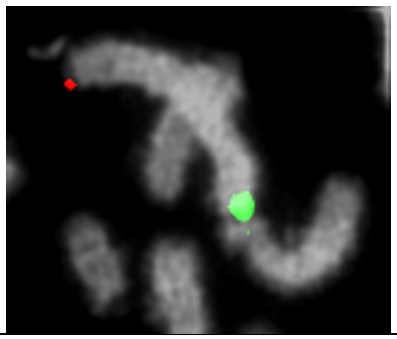
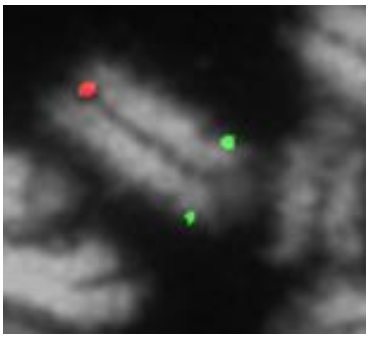
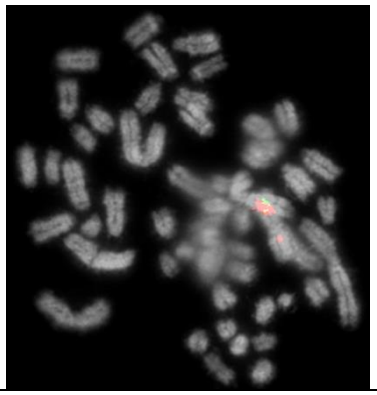
Table 4.2: Table illustrating whether or not cattle probes (FITC or Texas) targeting each chromosome below successfully hybridised to chromosomal position in a control cattle sample and a sheep sample. Boxes shown in black were not considered in this study.

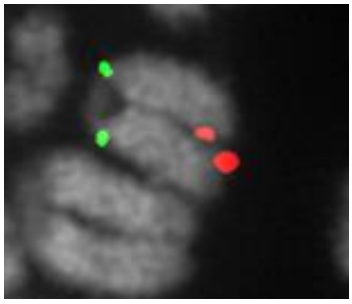
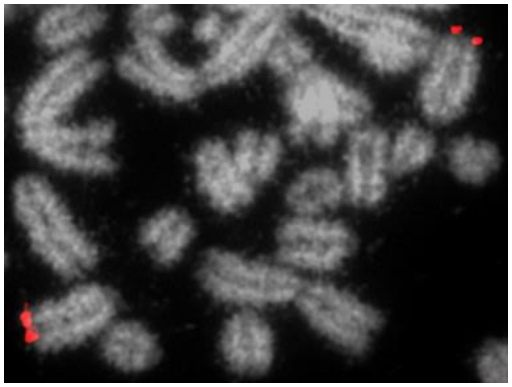
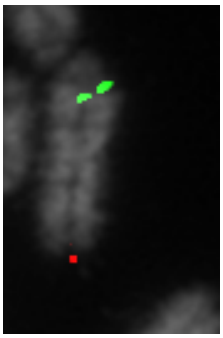
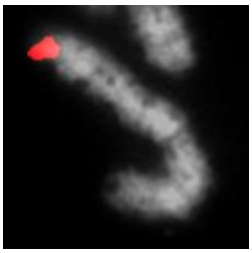
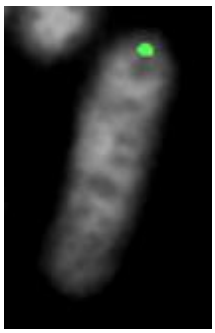
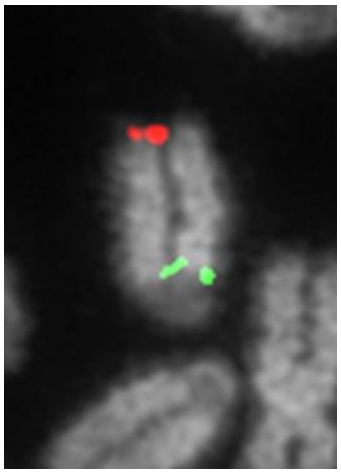
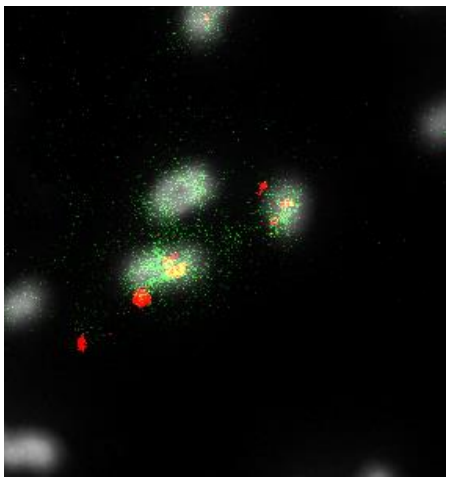
Chromosome	Control sample		Sheep sample	
	FITC	Texas	FITC	Texas
1	Yes	Yes	Yes	Yes
3	Yes	Yes	No	No
4	Yes	Yes	No	Yes
5	Yes	Yes	Yes	Yes
6	Yes	Yes	No	No
7	Yes	Yes	Yes	Yes
8	Yes	Yes	Yes	Yes
10	Yes	Yes	Yes	Yes
11	Yes	Yes	No	No
12	Yes	Yes	No	No
13	Yes	Yes	Yes	Yes
14	Yes	Yes	Yes	Yes
15	Yes	Yes	Yes	No
16	Yes	Yes	Yes	Yes
17	Yes	Yes	Yes	Yes
18	Yes	Yes	Yes	Yes
19	Yes	Yes	Yes	Yes
20	Yes	Yes	Yes	Yes
21	Yes	Yes	Yes	Yes
22	Yes	ND	Yes	ND
23	ND	Yes	ND	Yes
24	Yes	Yes	Yes	Yes
25	Yes	Yes	Yes	Yes
29	Yes	Yes	Yes	Yes
X	Yes	Yes	Yes	Yes

4.2 Hybridisation Images for Specific Aim 2

Table 4.3 shows how bovine subtelomeric probes hybridise to a cattle sample (control) for which they have been designed and proven to work for and how they hybridise to the sheep sample on the right hand column. Both chromosome 1 targetting probes successfully hybridised to the same sheep chromsome in the centromeric and subtelomeric positions. Neither chromsome 3 targetting probes had successful hybridisations in the sheep sample. the chromsome 4q (Texas) bovine probe successfully hybridised on a sheep chromosome in a subtelomeric position however, 4p (FITC) did not. Table 4.3 illustrates that bovine probes 5p and q both successfully hybridised onto ovine chromosomes in subtelmoeric positions. Chromsome 6 probes did not successfully hybridise on ovine chromomes. The pair of chromsome 7 bovine probes are shown to both succefully hybridise to subtelomeric positions on either end of the same chromosome. The chromosome 8 probes also both hybridised onto the same sheep chromosome with the FITC probe attaching to a centromeric region and the texas probe attaching to a subtelomeric region. Finally, chromosome 10 probes both successfully hybridised with either subtelomeric end of the same ovine chromosome.

Table 4.3: Images on the left-hand column show how probes normally attach to the chromosomes of the control (cattle). Images in the right column show the affinity of hybridisation of the same probes attached instead to sheep chromosomes. The position of probes on each chromosome is also shown. Images use a P (FITC/green) probe and a Q (Texas/red) probe for chromosomes 1, 3, 4, 5, 6, 7, 8, 9, 10 which have already been established in cattle which is shown in the left control image (control cattle sample) and tests these probes on ovine samples (right-hand column). For simplicity and to better illustrate the signals, where probes have clearly hybridised with strong signals only one chromosome is shown.

Probe	**Cattle**	**Sheep**
1p+q		 
3 p+q		No signalling present 

4 p+q		
5 p+q		FITC and Texas found in separate metaphases  
6 p+q		Signals not clear enough 

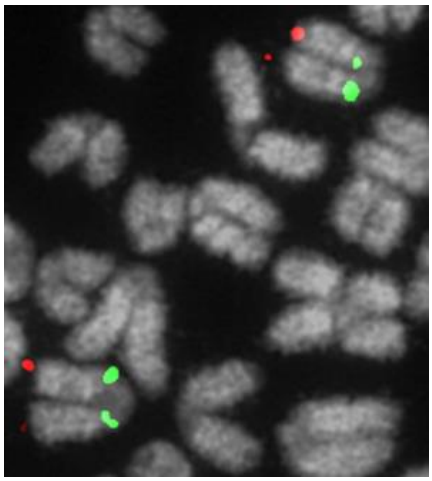
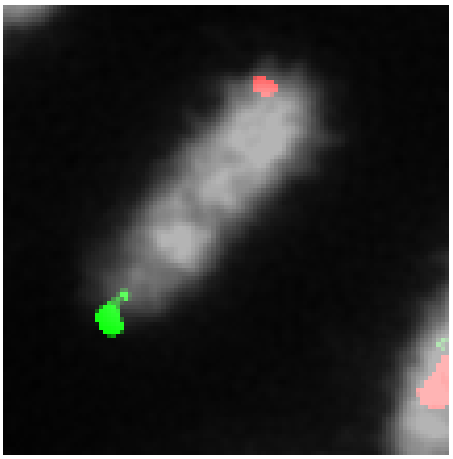
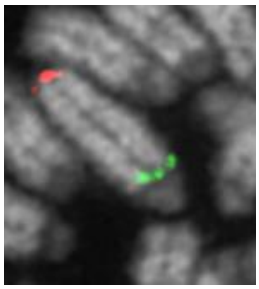
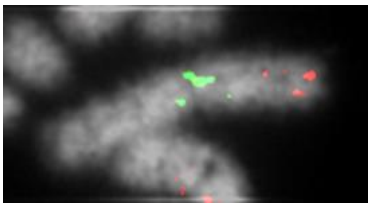
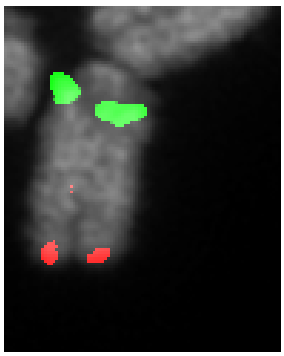
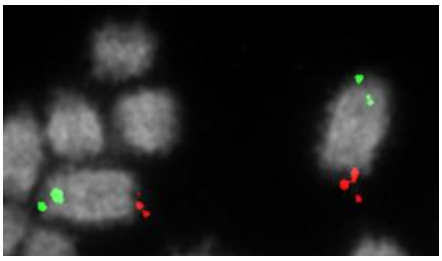
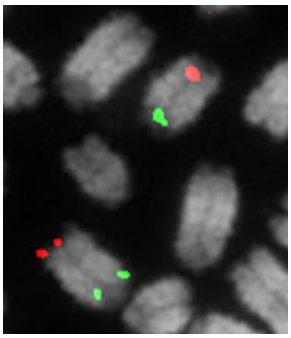
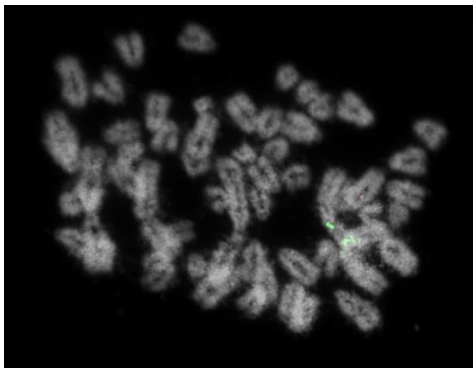
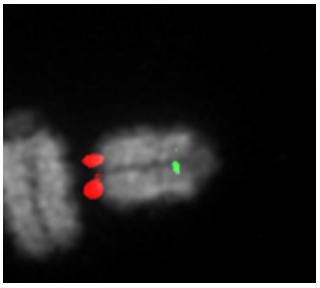
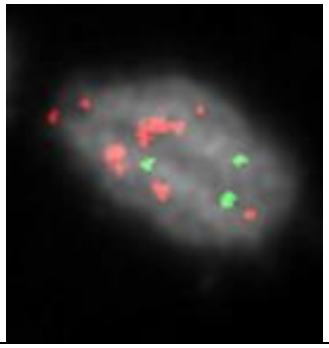
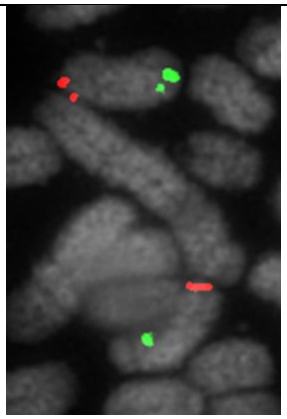
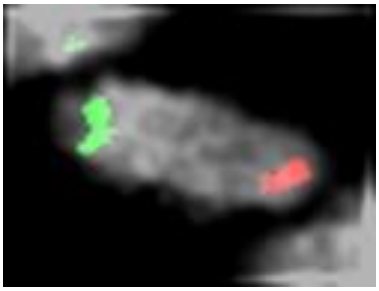
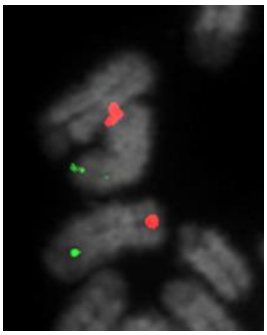
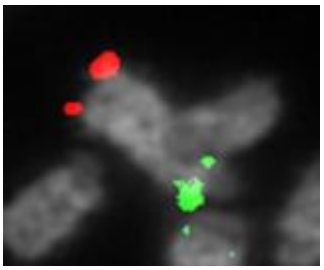
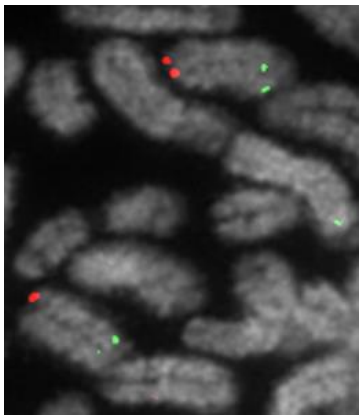
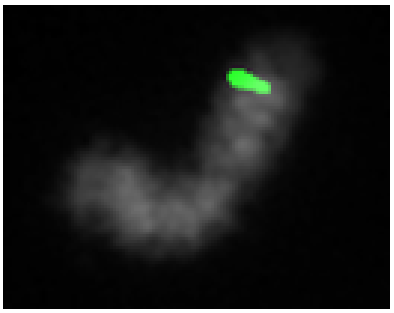
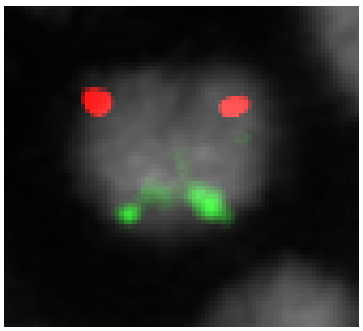
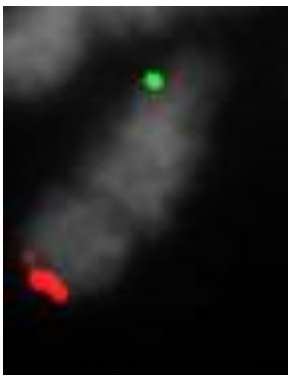
7 p+q		
8 p+q		
10 p+q		

Table 4.4 shows the intermediate sized chromsome targetting probes and their hybridisations which were largely successful. Bovine probe pairings which had two successfully hybridised probes were chromosomes 13, 14, 16, 17, 18 and 19. The bovine chromosome 15 probe pairing also had one successful hybridisation with the FITC probe which hybridised to a subtelomeric region. All successful paired probe hybridisations also bound to opposing subtelomeric regions of the same chromosome. The bovine probe pairings for chromosome 11 and 12 as well as Texas chromsome 15 probes demonstrated no successful hybridisations on ovine chromosomes.

Table 4.4: Images on the left-hand column show how probes normally attach to the chromosomes of the control (cattle). Images in the right column show the affinity of hybridisation of the same probes attached instead to sheep chromosomes. The position of probes on each chromosome is also shown. This table shows how probes for bovine chromosomes 11-19 bind to a control cattle sample (left) and an ovine sample (right). For simplicity and to better illustrate the signals, where probes have clearly hybridised with strong signals only one chromosome is shown.

Probe	**Cattle**	**Sheep**
11 p+q		No clear signals 
12 p+q		Too much off-signalling. 
13 p+q		

14 p+q		
15 p+q		FITC only 
16 p+q		

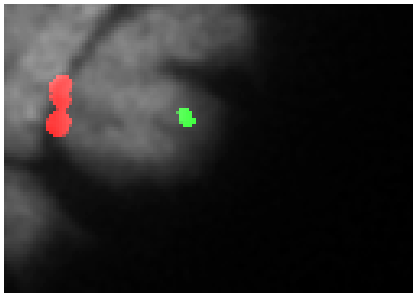
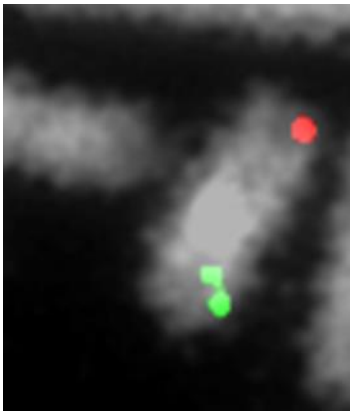
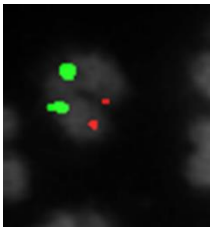
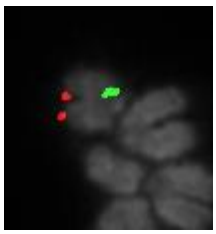
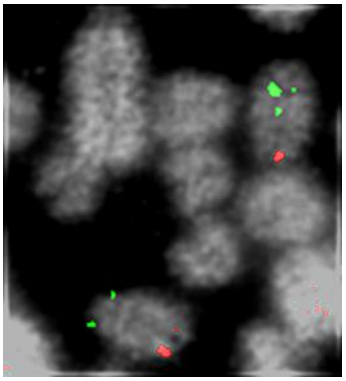
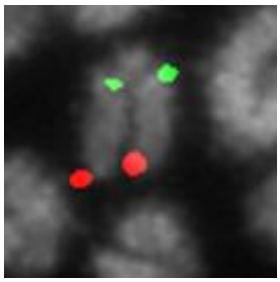
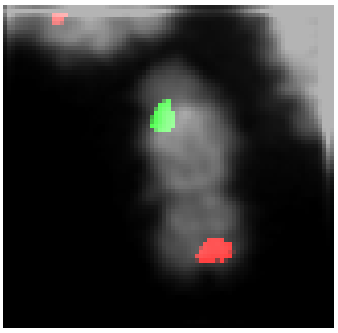
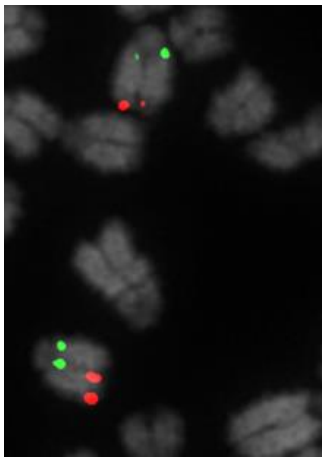
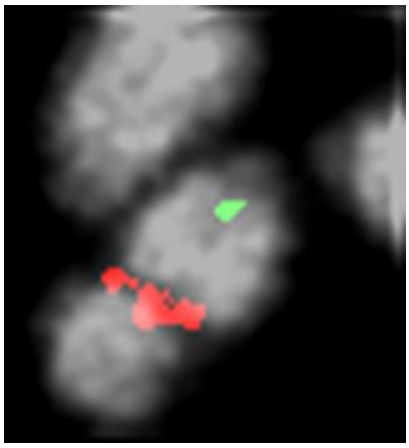
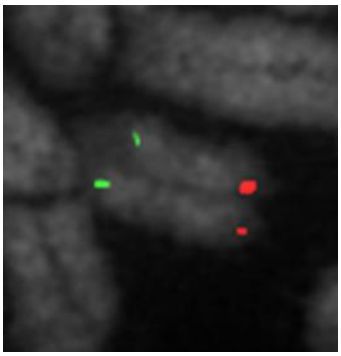
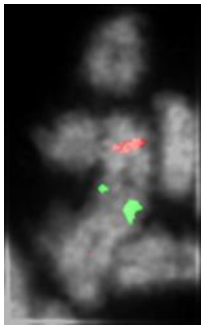
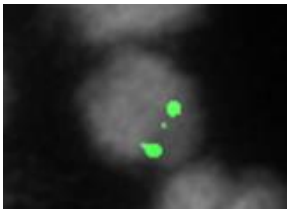
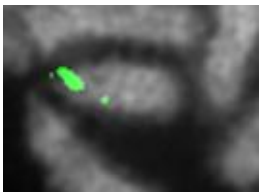
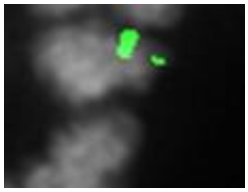
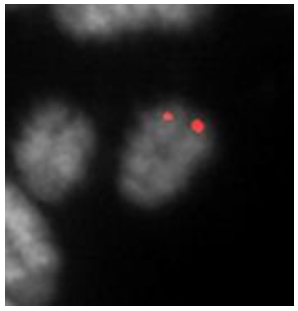
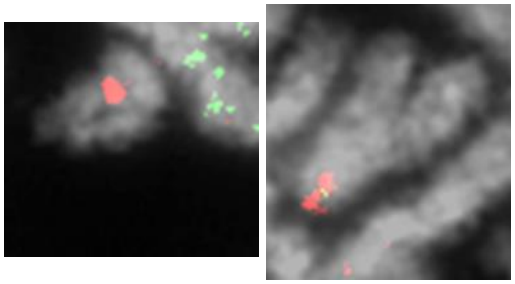
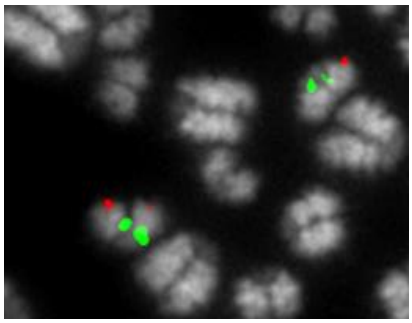
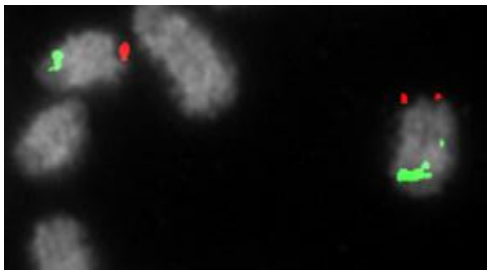
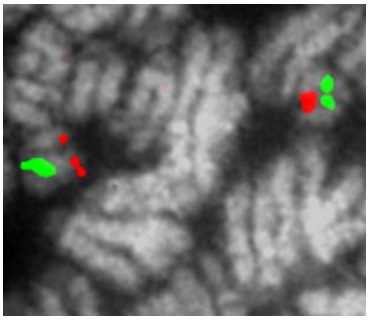
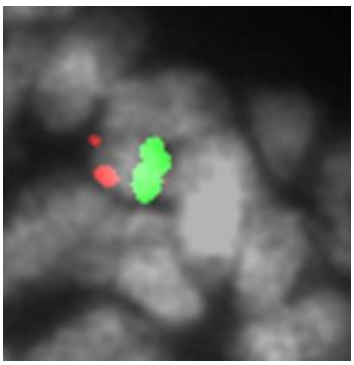
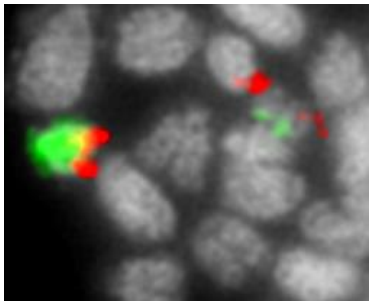
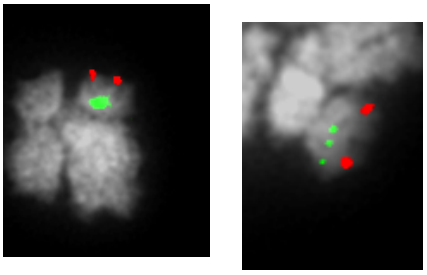
17 p+q		
18 p+q	 	
19 p+q		

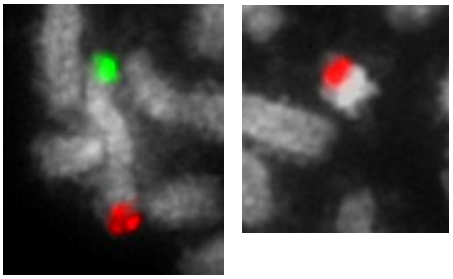
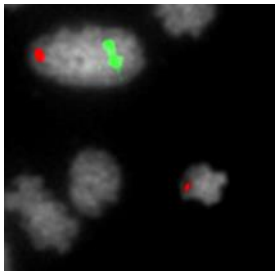
Table 4.5 shows how subtelomeric cattle probes hybridise to the cattle control sample on the left-hand column and ovine chromes on the right for probe pairings targeting cattle chromosomes 20 to 25, chromosomes 29 and X. The chromosome 20 probe pairing successfully hybridised onto the same ovine chromosome with the FITC probe hybridising into a subtelomeric position and the Texas probe hybridising into a centromeric position. Likewise, the chromosome 21 pairing both hybridised onto the same ovine chromosome with the FITC probe hybridising into a centromeric position and the Texas probe hybridising to a subtelomeric position.

Only one probe FITC or Texas of the usual pairing was available to test for chromosomes 22 and 23 respectively as shown in table 4.5. However, each of the probes that were available for each of these chromosomes hybridised and both of them hybridised in subtelomeric positions. The chromosome 24 bovine probe pair strongly hybridised to the same ovine chromosome and in opposing subtelomeric positions. Chromosome 25 bovine probes also hybridised successfully to the same ovine chromosome and potentially in subtelomeric positions although whether these are definitely subtelomeric positions is not clear in the image in table 4.5. The probe pairings for bovine chromosome 29 successfully hybridised onto the same ovine chromosome in the centromeric (FITC) and subtelomeric positions (Texas). Finally, X targeting bovine probes successfully hybridised on the same sheep chromosome which is likely the sheep X chromosome and both probes hybridised to opposing subtelomeric ends of this chromosome.

Table 4.5: Images on the left-hand column show how probes normally attach to the chromosomes of the control (cattle). Images in the right column show the affinity of hybridisation of the same probes attached instead to sheep chromosomes. The position of probes on each chromosome is also shown.
This figure shows how probes for bovine chromosomes 20, 21, 22, 23, 24, 25, 29 and X bind to a control cattle sample (left) and an ovine sample (right).
For simplicity and to better illustrate the signals, where probes have clearly hybridised with strong signals only one chromosome is shown.

Probe	Cattle	Sheep
20 p+q		
21 p+q		
22 p		 

23 q		
24 p+q		
25 p+q		
29 p+q		

X p+q		

In total, of the 48 probes counted in this study 38 gave strong clear signals which were suitable for a diagnostic device. In all succesful hybridisations, probes appeared to bind to regions either at the end of chromosomes in the subtelomeric regions or centromeric positions wherever the centromere may be. 33 of 48 (69%) of the probes which were used in this study bound to a position which is likely a subtelomeric region (end of chromosome). 24 of these made 12 complete pairings where probes attached to regions within in each end of the chromosome which can be assumed to be in the subtelomeric regions. To better understand which chromosome the probes have attached to a karyotype would need to be undertaken for each pairing to number each sheep chromosome and deduce which chromosomes the hybridisations have occurred.

In 5 cases of probes appeared to hybridise to positions more centrally in the chromosome. This only occurred in the 3 submetacentic chromosomes as the probes hybridised within the centromeric regions in all of these cases. Interestingly, this seemed to only occur with probes that target the larger (chr1, chr8) or smaller (chr20, chr21, chr29) chromosomes. This makes sense as submetacentric chromosomes have short p arms and long q arms. The rest of the probes failed to succesfully hybridise. Most of the probes that failed hybridisation did have visible signals however, these were not strong enough to be considered for a sheep device and would often

come with high amounts of off-target signalling in other regions of the metaphase. Most imaged probes had a high affinity with the sheeps chromosomes as they very strong/bright signals.

The results demonstrated strong signals in subtelomeric regions of the X chromosome which is confirmed by the presence of texas signals on the nearby Y chromosome as shown above.

5: Discussion

5.1: Specific aim 1

5.1.1 Pig screening

The period 01/2021-05/2022 in which the above data was collected was a period heavily affected by the COVID-19 pandemic. Typically, for samples to be high enough quality for use in either FISH or karyotyping, blood extraction should be undertaken maximumly 1 day before culturing. Severe delays in deliveries due to the pandemic, meant that batches of blood samples would often arrive 3-4 days later than expected leading to many blood samples failing culture and often sub-optimal quality in the rest of the samples. These sub-optimal samples would often first have very low mitotic indexes meaning there were less available metaphases to analyse. Secondly, chromosomes in available metaphases in samples which had been cultured 2-4 days late were found to be of low-quality (unclear and fuzzy) meaning karyotypes would have often been almost impossible to carry out due to difficulties in manually differentiating each chromosome. This does however demonstrate perhaps the greatest advantage of FISH over karyotyping, as probes were still able to bind to unclear chromosomes and as long as two homologous chromosomes which had probes bound either side were present in each box of an individual FISH slide, we could still confirm the diagnosis of each individual pig.

The collective data illustrated an unprecedented proportion of 14.5% of screened pigs being diagnosed with an abnormality (table 3.1). This inflated figure is largely down to one translocation namely RT(1;2) which ~13% of screened pigs were found to be a carrier of. This is presumed to be due to largely be down to the unavailability of labs during closures during the COVID-19 pandemic. It is likely that an unscreened *de novo*

boar was used in the AI of many sows and this translocation was then passed down to many offspring. The company for which this population originated reported a significant reduction in litter size and farrowing rates. From January 2021, 100 cases were identified over several months by FISH of this translocation and reports of carriers were sent back to the company which eventually lead to successful elimination of the translation from the breeding population by May 2021.

This translocation appeared to be too small for a standard karyotype to pick up even with high quality chromosomes as the difference in size between the normal and translocated chromosomes 1 appear indistinguishable. However, as reported by the company providing the samples, this translocation had a confirmed negative effect on the fertility of carriers. Therefore, this highlights the benefits of FISH over karyotyping as FISH was sensitive enough to consistently pick up this translocation. Without FISH the origin of the reduced fertility in this population may have not been found and the population may have been indiscriminately culled rather than carrier individuals being found.

The RT(9;18) translocation occurred in the same population of pigs in which the RT(1;2) translocation occurred. All 4 cases of this translocation occurred within the same batch which also contained 16 RT(1:2) individuals. There were no individuals found who had both translocation the translocation existing within the same population. This suggests that being a carrier of both translocations may not be compatible with life. No basic karyotype could be made due to lack of high enough quality metaphases being found. This translocation has however been previously reported (60) through karyotyping. Although the translocation we discovered could be a different translocation between the same chromosomes, the translocation that has been reported would suggest that a karyotype is sufficient to diagnose this

translocation in normal circumstances when the metaphase is high enough quality to do so.

RT(1;3) was an isolated case and was found before the individual was utilised for breeding therefore the effect on fertility could not be observed. Due to the relative momentous size of this translocation as shown in the karyotype in figure 3.6 one would hypothesise that this translocation would cause a significantly increased likelihood of unbalanced gamete production and therefore reduced fertility.

RT(9;10) was an isolated *de novo* case of a previously reported rare translocation. Once again, a karyotype could not be made due to the boar sample being sub-optimal sample however, it was of high enough quality to perform FISH. Likewise, reciprocal translocation RT(7;17) and RT(10;17) were both *de novo* cases of rare translocations which had previously been reported by use of a karyotype suggesting a karyotype may be sensitive enough to detect these translocations(62,63). That is if translocations we discovered on the same chromosomes are the same exact translocations that occurred in these studies. In neither of these cases a karyotype could be made but FISH analysis could be untaken demonstrating strong signals in target chromosomes (Figure 3.9).

In the period in which this data was collected two pigs were found that presented with metaphases and interphases with both 1 X chromosomes and 2 X chromosomes as shown in figure 3.10. As porcine Y chromosomes do not bind to X targeting probes, without a karyotype of both an XX presenting metaphase and XY presenting metaphase we cannot be certain of whether or not this individual is XX/XY chimeric or XXY/XY mosaic. In this regard the karyotype may have an advantage over a FISH analysis. Reciprocal translocations although common are relatively distributed in

terms of prevalence of specific translocations. Whereas chimerism/mosaicism has consistently appeared in many studies albeit with a low prevalence.

The work done in the screening of boars following the initial creation of the Porcine Multiprobe® device between 2016 and 2019 demonstrated that 4.5% of screened pigs presented with a chromosomal abnormality but due to many pigs being clearly directly related an estimated incidence of 0.88% (46) of pigs with a *de novo* balanced reciprocal translocation (RT) was found. This data clearly demonstrated a large increase then previously the reported incidence of 0.47% (63). Our data does not demonstrate as high of an incidence at 0.77% as initial results suggested published using the Multiprobe® device at 0.88%. However, my results did still show a marked increase in incidence than when only karyotyping was used and indeed showed a far more similar value to that previously reported in my lab. Our data therefore further supports the previous study untaken by our lab which reported the high likelihood that *de novo* RT incidence had been previously underestimated at 0.47%.

5.1.2 Cattle screening

The lab received 3 bull samples in the period between January 2021 until May 2022. These specific bulls will have had significant value due to the fact 1 breeding bulls used to artificially inseminate potentially >100,000 cows. These bulls were also selected by breeders with prior suspicion or knowledge of a particular aberration however, samples were sent for confirmation of a specific diagnosis.

By far the most prevalent aberration in cattle is the RobT(1;29) translocation which along with other Robertsonian translocations is very easy to spot without using fluorescent probes or creating a karyotype. However, to determine the chromosomes involved in a Robertsonian translocation either method can be used. Karyotyping must

be performed fully with banding techniques to correctly determine which chromosomes are involved due to inaccuracies that will likely arise in differentiating between cattle chromosomes purely by size. FISH offers a far quicker easier solution to this using a Cattle Multiprobe® device to quickly determine which probes attach to the Robertsonian fusion and therefore the chromosomes that are involved.

The first aberration that was located in cattle was a RobT(1;29) which could be seen clearly using both FISH or a karyotype. The karyotype was made using the image taken from our FISH analysis. Without banding in the karyotype there would not have been reasonable evidence to prove the chromosome fused with chromosome 1 was in fact chromosome 29 and not chromosome 28 which is nearly identical in size as shown in figure 3.12. This is not an issue when using FISH.

The other aberration found in cattle was an aneuploid XXY individual. This diagnosis could have equally been made using a karyotype due to the obvious addition of a chromosome especially the X chromosome which is the only submetacentric chromosome in cattle.

The cattle device is still a fairly novel development and has not yet been utilised as much as our porcine device. The samples in which I screened were likely from individuals already presenting with a developmental issue or subfertility therefore were a bad indicator of the larger world-wide cattle population as a whole. Previous work in the development of the Bovine Multiprobe® device argued that the prevalence of reciprocal translocations in cattle are likely grossly under reported. A study from 2008 found a reporting rate of 0.03% of cattle with a RT (64). This is partly because these translocations are often overshadowed by Robertsonian translocations which are relatively easy to detect as they cause a dramatic change and are common in cattle

(35). With cattle chromosomes being all acrocentric and relatively similar in size the need for a cattle device to find smaller more cryptic translocations is almost certainly higher than anticipated. Our study did not demonstrate this however, and indeed the small number of cattle samples received for this study (three) was insufficient data to research this further. Instead, the few samples which were provided could have easily been diagnosed by a basic karyotype or a chromosomal count.

5.1.3 Conclusions for Specific aim 1

Cytogenetic tools such as FISH are locating more than previously expected translocations due to an increased sensitivity in detection over karyotyping. This may have enormous financial benefit to the livestock industry. By locating chromosomal aberrations carriers can be located before semen extraction for AI and removed from the breeding population of boars. Without these cytogenetic tools drops in litter size or farrowing/calving rates would occur without explanation and would likely have a significant financial burden . As can be seen in figure 3.2 without locating and removing *de novo* carriers of a translocation from a population before breeding the translocation can ultimately be passed down to offspring and the number of sub-fertile individuals can multiple. This causes a significantly larger financial burden for breeding companies as they are faced with the dilemma of either removing a significantly higher number of bulls intended for breeding from the population or, facing a reduction in littering rates which ultimately will cause a significant loss of efficiency and resources. These results have further shown the strong advantage of FISH over karyotyping. FISH offers a quicker, easier, more sensitive method with far higher success rates in reliable diagnosis. During COVID a drop in sample quality would have made karyotyping too difficult to perform well whereas this was shown to not be an issue when carrying out a FISH analysis using a Multiprobe® device.

5.2: Specific Aim 2

5.2.1 Ovine Multiprobe Device production

As cattle devices had not been produced in a long period of time before this experiment was undertaken new devices had to be produced. Probes stocks also needed replenishing as many probes solutions had run out as well as a few solutions showing signs of contamination of microorganisms. Contamination meant that it was extremely likely that the probes in these solutions would have been digested and therefore also needed replenishing. Probes stocks were replenished by growth of BACs from glycerol stocks and subsequent amplification, fluorescent labelling and purification of probes. Despite replenished probe solutions working very well, some of the original probes stock solutions were no longer working when tested on the control sample. These solutions were not originally checked to prevent waste of probes which are labelled with expensive FITC and Texas dyes. This did however mean that 12 out of 60 probes were not tested as they did not appear on the control sample. Despite this probes that did work often gave very promising results in their binding to sheep chromosomes.

38 of the 48 tested probes hybridised showing strong clear signals which could be potentially used in a novel sheep device if demand for such a device should arise in the future. This will save a lot of time and resources spent in otherwise locating suitable regions in the sub-telomeres of sheep chromosomes and BAC selection. Even if not all ovine (sheep) chromosomes have a pair of cattle probes that bind in the p and q arms, as a significant proportion of chromosomes do have probe hybridisations present, time and resources can still be saved. Out of the 27 chromosomes in the ovine diploid set, 12 complete sub-telomeric pairings (24 paired subtelomeric probes) were found using the cattle probes. Although karyotypes were not made to see which

chromosomes the translocations occurred on, one would hypothesis that each of these pairings are on different chromosomes as this is the case on the cattle chromosomes. Cattle and sheep are relatively closely related and it would be unlikely that the conserved region of both ends of a chromosome translocated to the same chromosome. In fact, whenever both probes (p (FITC) and q (Texas)) for a given cattle chromosome hybridised on a sheep metaphase, both hybridisations always occurred on the same chromosome. Due to slight changes in an ovine chromosome set compared with bovine chromosomes these probe pairings may have occurred on chromosomes of differing chromosome numbers e.g., cattle 13 probes may hybridise to chromosome 15 in sheep. This can only be determined by performing a karyotype in each sheep metaphase where a pair of probes has hybridised to a specific chromosome.

An additional 9 sub-telomeric probes, demonstrated strong signals however they were unpaired to another sub-telomeric probes (p and q). These could also be used in producing an ovine device however, new probes would need to be found for the opposing subtelomeric end. In 5 of the chromosomes where this was the case, with 1 subtelomeric probe bound, a second paring probe was present however in all these cases the pairing probe was found in a centromeric position and therefore were not useful in a clinical probe. With the remaining unpaired subtelomeric probes, a second signal was not present which may be caused by a number of factors: an issue with the probe sample quality, an error with the FISH process or the specific probe did not hybridise well with the corresponding ovine chromosome.

These remaining hybridised probes gave a fascinating unforeseen insight into chromosome evolution between cattle and sheep chromosomes. When comparing a basic unbanded karyotype between the two species one would notice that the first 3

ovine chromosomes are submetacentric compared to cattle which are all acrocentric. The next obvious observation would be the addition of three more pairs of homologous chromosomes in the cattle karyotype compared to the sheep. The results found show that these differences between the ovine and bovine chromosome set may be linked. The 5 cases, where sub-telomeric cattle probes appeared to hybridise in centromeric regions of the ovine submetacentric chromosomes may suggest that 1) Multiple fusion events (e.g., Robertsonian translocations) between non-homologous chromosomes of a common ancestor's (of both sheep and cattle) set of chromosomes lead to the modern sheep's karyotype while these fusions did not occur in the cattle. Or 2) multiple breakages in a common ancestor with a karyotype more closely resembling the sheep's karyotype led to the evolution of the modern cattle karyotype. To further support this hypothesis the probes which hybridised to centromeric positions of the ovine submetacentric chromosomes typically targeted the smaller cattle chromosomes (chromosomes 20, 21 and 29) and the larger ones (chromosomes 1 and 8). This is expected as the sheep chromosomes in which these probes hybridised were submetacentric meaning the short arms are considerably shorter than the long arms. As shown in Figure 1.9 and 1.10 bovine and ovine karyotypes are very similar with the exception of the submetacentric chromosomes in sheep.

Previously, our lab had produced a clinical Porcine and Bovine Multiprobe® (35, 46) device which have already shown promising results and has been attracting significant usage from large breeding companies across Europe especially with our porcine device. Further to this, other work which inspired this project included a study which located highly conserved regions of avian chromosomes, using these to find BACs which were used to produce universally hybridising FISH pan-avian probes (65). There probes were then be used in a subsequent project where they were used to map

chromosomal evolutionary rearrangements between distantly and closely related species (66). Universal pan-avian probes worked as the probe target regions are so highly conserved across all avian species that probes were able to not only bind to every species but also some reptilian species (65). With this in mind and the knowledge that the chromosomal regions of the subtelomeres targeted with the FISH cattle device were also extremely highly conserved and in simple non-coding repeat regions like the ones used in the pan-avian probes it was reasonable to believe that the preselected bovine BACs used for the bovine multiprobe device may also work on sheep chromosomes who are not just part of the same class (Mammalia) but also the same order, artiodactyl (even-toed ungulates). It made sense to hypothesise that the bovine probes would have sufficient efficiency on ovine chromosomes for an ovine device however this did not mean that the bovine probes if successfully hybridised would be in a suitable position for clinical use. As chromosomes evolve and there is a clear visible difference due to evolution between ovine and bovine chromosomes the conserved regions used for chromosomal analysis can be shuffled into positions no longer in the subtelomeres of target chromosomes and may commonly for example be found in the centromeric region of the chromosome. We believed however, that substantial time and resources would have been saved by using preselected cattle probes to attempt to produce a sheep device and at least reduce the number of new probes which needed preselecting as well as producing.

5.2.2 Conclusions for specific aim 2

These results demonstrate that there is a great potential for the production of a sheep device using probes designed for a Cattle Multiprobe® device. This study has shown that many ovine chromosomes will likely already have paired sub-telomeric probes purely from using the existing library of bovine sub-telomeric probes. Moreover, the

data likely underestimated the number of bovine probes which bind to subtelomeric regions of sheep chromosomes. This is because 12 probes that may have potentially hybridised to these regions were not included in this study. Furthermore, only one sheep sample was used, and this sample was not used in any repeats. This sample was over a decade old and may not have given accurate results. Repeats or additional sheep samples might have shown additional hybridisations which were not originally present.

This study also reported on potential evolutionary basis behind chromosomal differences between the ovine and bovine karyotypes.

6: General discussion

Our results demonstrate the high level of sensitivity of detection and low rates of error when using a FISH Multiprobe® device system. This could be of massive benefit to a huge global breeding industry which rely on a few high-quality breeding males to artificially inseminate potentially thousands of females. Allowing a translocation to spread over a population so that there is a significant drop in fertility across the whole population could cause detrimental financial loss to breeding companies. It is therefore of great importance, in the modern climate where meat consumption is at an all-time high to screen animals for aberrations before they are used for breeding. This thesis has shown that FISH is far better option at achieving this compared with karyotyping, which is slower, cannot be performed well unless samples are of high quality and is not as sensitive and accurate in translocation detection specifically reciprocal translocations. Despite a period when sample quality was very low, 114 samples out of 778 total (porcine and bovine) exhibited aberrations. These were detected using the multiprobe device demonstrating high levels of affinity to the target and bright signals.

Many of these were reciprocal translocations some of which would have been undetectable by karyotyping.

Reciprocal translocations in pigs were first recorded with cytogenetic screening techniques in pigs in 1964 (67) using an unbanded karyotype while screening semen from a population of pigs with severely reduced fertility. Since this point over 200 different reciprocal translocations have been described in screening studies for almost 6 decades (62, 63, 67, 68-73). Robertsonian translocations although have been recorded in the domestic pig are very rare(64). This is likely due to Robertsonian translocations primarily occurring between acrocentric chromosomes and because pigs have far fewer acrocentric chromosomes in their Karyotype(57).

The continued work in porcine screening has shown great promise in consistently locating *de novo* translocations. Data provided from porcine Multiprobe FISH screening in this thesis and previous data collected using the Multiprobe device (46) show give an overall incidence of reciprocal translocations of 0.87% which is far greater than the previously appreciated 0.47%(63). An issue with this value is that because incidence is a relatively small number the sample size needed to validate this number as significant requires a much larger sample size. For this reason, with a 95% confidence level, the confidence interval calculated ranges between 0.19% to 1.56%. We calculated that to achieve a confidence interval with a margin of error +/-0.05% we would need to screen ~1,500,000 samples. Screening work must therefore continue for a significant period before a higher confidence in the incidence of *de novo* reciprocal translocations can be confirmed. However, this value does not consider the consistent findings of translocations over a 6-year period and de novo translocations being found in completely random and independent populations from across Europe using the Multiprobe® FISH method. The actual number of reciprocal translocation

carriers was also far greater in both studies which may also be taken into consideration. Over this period, we have consistently found an incidence value between 0.75% and 0.95% which is almost double the incidence value found by Giesma-stain karyotypes(64).

After the discovery of the common translocation Rob(1;29) in Swedish Red cattle breeds in 1964 (74), and its harmful effect on fertility, the application of clinical cytogenetics in livestock mammals has widely expanded with the intent on finding abnormalities and their effects on fertility. Discoveries of new Robertsonian translocations have been commonplace since the 1970s with new translocations having been found regularly since that point using karyotypes in both sheep and cattle(74-84). Interestingly, all Robertsonian translocations found in cattle have been dicentric with the exception of Rob(1;29) (40). C-banding or G-banding karyotyping which have been commonly used in the majority of livestock cytogenetic screening studies clearly show this as centromeres are clearly visible with these stains due to them being dense areas of heterochromatin(9, 56). G/C-staining Karyotypes have been a valuable and sufficient tool in locating Robertsonian translocations but have likely been a poor tool for locating Reciprocal translocations which are often too small to detect using karyotypes and likely has an even greater negative effect on the fertility of carriers(85).

Up until recently reciprocal translocations have been thought to be a rare occurrence in cattle, with only 20 cases being reported up until 2020 (35, 85, 87-92). The notion that Robertsonian translocations are more common that reciprocal translocations and reciprocal translocations are rare in cattle have long been accepted. An article published in 2011 (85) used a mathematical approach to calculate an estimation of the theoretical incidence of reciprocal translocations in cattle the study used the Italian

official Cytogenetic screening programme where 22,735 animals were screened over a 15-year period. In this time 5 carriers of reciprocal translocations were detected using the standard Giemsa staining method. This study took this value for carriers of translocations detected and combined it with an estimation value for number of carriers which would have theoretically gone undetected. Undetected carriers were estimated through many important factors such as theoretical breakpoints and importantly the knowledge that Giemsa banding techniques are only able to detect reciprocal translocations which are at least one derivative longer than M (a value given in this study). The value given for the estimated incidence of reciprocal translocations in this study was 0.14% which is a value 5 times higher than is shown for *de novo* Robertsonian translocations (85). This value although an estimation gives precedence to our work in the cattle Multiprobe device. The main advantage the Multiprobe® FISH device has over Giemsa karyotyping in cattle is being able to locate all reciprocal translocations which have likely been severely underreported and may have a greater impact on fertility than Robertsonian Translocations.

The strength of the porcine and bovine device shown by the results further display the potential for a multiprobe device in sheep. My results show that a sheep device may in large part be designed using the cattle BAC library as 79% of tested cattle probes hybridised with clear, bright signals on ovine chromosomes. Many of these were already in subtelomeric regions ready for use in a device. The position of each probe does however need confirmed by way of individual karyotypes.

Incidences of chromosomal abnormalities in the domestic sheep were first documented in the early 1970s firstly with cases of Robertsonian Translocations(75), since ~7 different Robertsonian translocations have been found in sheep(75, 76, 77, 78, 79, 86). As studies in sheep have been so rare it is difficult to make an estimate

into the incidence of these abnormalities. Less than 10 *de novo* reciprocal translocations have been documented in sheep thus far (64, 93-99) all cases had led to severe loss in fertility in carriers. However, once again cytogenetic screening studies have often overlooked sheep compared to cattle and pigs possibly because they are less economically productive in terms of meat/dairy and other production(2). This should not be a deterrent for further research in producing a sheep device as sheep and cattle are relatively closely related species (bovids) meaning time and money will be saved not having to produce a completely new set of probes. Similar to in cattle and pigs it is extremely likely, occurrence of RTs have been grossly underestimated and likely play a significant role in subfertility. Incidences of reciprocal translocations, and Robertsonian translocations have been steadily documented in domestic sheep over the past 20-30 years however it would be very difficult to draw conclusions of the incidence of these abnormalities from specific occurrences in various studies(64, 75-79, 85, 93-99).

A limitation in specific aim 1, was that although the sample quality acceptable for FISH this could have been limiting in the reliability of FISH. Samples with mitotic indexes too low to screen often had to be discarded as FISH would not be possible. The data is also not a good reflection of actual incidence/prevalence of aberrations in livestock as a whole as often the aberration would cause a noticeable drop in fertility in the carriers, which may have acted a driver for the company to send samples to our lab for diagnosis. The extent in which this may have happened is unknown due to lack of information given to our lab regarding the samples which they have provided us. Another limiting factor of specific aim 1 is that because incidence of reciprocal translocations is so low even with the upper estimates, many more animals would need to be screened to confirm a more accurate value for prevalence of RTs.

For specific aim 2, the main limiting factor was the lack of probes that worked on the control sample and therefore needed to be disregarded. A fresh stock of probes was needed as probes appeared to have degraded after not being used for a long period of time. This meant that an incomplete device was used in the collection of the data for this section. Furthermore, more than one sheep samples preferably a more recently taken one should have been used to make the experiment more reliable and comprehensive.

7: Conclusion

To conclude, screening of mammalian livestock for chromosomal aberrations is an area which will likely have a more than enough of a financial benefit to warrant the routine screening of males before semen extraction for AI. Despite a close true value for incidence of reciprocal translocations and Robertsonian translocations not being found we have demonstrated that incidence of *de novo* translocations estimations previously made though cytogenetic studies which used the standard Giemsa Karyotyping method are likely gross underestimations of the true incidence. Furthermore, financial loss from the introduction and potential spread of a single undetected *de novo* translocation into a population will have a far larger financial burden than the relatively low cost of screening. The best and most effective way to screen for chromosomal aberrations is through Multiprobe FISH. Existing Multiprobe devices in pigs and cattle are highly accurate and show clear and strong signals consistently. Therefore, there is potential for a novel ovine device to screen sheep for chromosomal aberrations. Application of our Cattle Multiprobe® device on a sheep sample show that a sheep Multiprobe® device could potentially be entirely produced using cattle subtelomeric FISH probes.

8: Future work

Specific aim 1 (a and b) was to screen porcine and bovine samples for chromosomal abnormalities characterising any novel translocations and to work out the prevalence of *de novo* translocations in each species. In this respect I believe that these studies should be continued in the years to come as our devices our more sensitive at picking up translocations then previously used methods novel translocations are likely to be found in each species in the coming years. A continuation of these studies will also help produce a more precise estimation for the overall incidence of reciprocal and Robertsonian translocations in cattle and pigs and soon maybe sheep as well. This information could be great use for example in the livestock industry or from an evolutionary standpoint as it may give an indication to the rate of chromosomal evolution. The second element of specific aims 1a and b was whether a karyotype was a sufficient clinical cytogenetic tool in locating chromosomal abnormalities. We demonstrated that karyotyping was a far inferior tool in this respect, therefore this element of specific aim 1 can be concluded.

Future work into specific aim 2 is to complete the sheep device by firstly, mapping the exact locations of subtelomeric cattle probes on each chromosome which can be done by using a combination of FISH and karyotyping techniques. Secondly, by finding potential matches for any remaining subtelomeric probes needed for a complete device.

Finally additional work could be production of a multiprobe device for other mammalian species where breeding is very regulated.

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10: Appendix

Chrom	Arm	Clone Name	Span	Chrom	Arm	Clone Name	Span (bp)
1	p	CH240-321O2	179,965	16	p	CH240-139M7	166,377
	d	CH240-96M6	187,920		d	CH240-315I10	186,228
2	p	CH240-457J20	198,157	17	p	CH240-267P22	176,654
	d	CH240-227E16	179,789		d	CH240-313I20	182,729
3	p	CH240-154A5	174,225	18	p	CH240-14C14	163,878
	d	CH240-302G6	190,291		d	CH240-436N22	179,260
4	p	CH240-416O20	170,609	19	p	CH240-349G17	169,018
	d	CH240-193F3	179,112		d	CH240-390C5	180,283
5	p	CH240-326L8	188,525	20	p	CH240-394L14	182,595
	d	CH240-248M21	163,993		d	CH240-339K22	183,557

6	p	CH240-324B6	180,970	21	p	CH240-301D14	163,699
	d	CH240-5F18	184,848		d	CH240-62O23	176,169
7	p	CH240-415D2	182,547	22	p	CH240-426O23	182,818
	d	CH240-276L16	168,781		d	CH240-313B20	173,299
8	p	CH240-443K7	175,465	23	p	CH240-102P19	179,615
	d	CH240-241A18	176,318		d	CH240-374G6	174,942
9	p	CH240-25A3	177,086	24	p	CH240-382F1	171,530
	d	CH240-298I24	172,331		d	CH240-19L13	171,917
10	p	CH240-421B11	166,378	25	p	CH240-198J4	186,545
	d	CH240-325F16	179,292		d	CH240-379D22	163,818
11	p	CH240-314K5	165,445	26	p	CH240-428I10	181,997
	d	CH240-344O3	183,795		d	CH240-389H1	176,691

12	p	CH240-261C16	164,440	27	p	CH240-7G11	184,155
	d	CH240-262C4	165,223		d	CH240-352M8	184,694
13	p	CH240-461F6	188,788	28	p	CH240-313L4	181,707
	d	CH240-471M8	178,736		d	CH240-63D12	183,932
14	p	CH240-319C15	181,738	29	p	CH240-367D17	179,713
	d	CH240-240M1	178,587		d	CH240-257F23	188,054
15	p	CH240-225A24	151,902	X	p	CH240-121E1	176,736
	d	CH240-386C2	168,728		q	CH240-472J20	186,872

Supplementary Table 10.1: Cattle BACs by chromosome from the CHORI-240 library used in the production of the cattle Multiprobe® device.

Chrom	Arm	Clone Name	Chrom	Arm	Clone Name
1	p	CH242-248F13	10	q	CH242-517L16
1	q	CH242-151E10	11	p	PigE-211E21
2	p	PigE-8G19	11	q	CH242-239O11
2	q	CH242-294F6	12	p	PigE-253K5
3	p	PigE-168G22	12	q	PigE-124G15
3	q	CH242-315N8	13	P	PigE-197C11
4	p	PigE-131J18	13	q	PigE-179J15
4	q	PigE-85G21	14	p	PigE-137C12
5	p	PigE-74P10	14	q	PigE-167E18
5	q	CH242-63B20	15	p	PigE-90C11
6	p	PigE-238J17	15	q	CH242-170N3
6	q	CH242-510F2	16	p	PigE-149F10
7	p	PigE-52L22	16	q	CH242-42L16
7	q	CH242-103I13	17	p	CH242-70L7
8	p	PigE-2N1	17	q	CH242-243H19
8	q	PigE-118B21	18	p	PigE-253N22
9	p	CH242-65G4	18	q	PigE-202I11
9	q	CH242-411M8	X	p	CH242-19N1
10	p	CH242-451I23	X	q	CH242-305A15

Supplementary Table 10.2: Porcine BACs by chromosome from the PigE-BAC library and the CHORI-242 Porcine BAC library used in the production of the porcine Multiprobe® device.