UNIVERSITI PUTRA MALAYSIA

THE USE OF ZINC FINGER PROTEIN GENE TO DETECT THE PRESENCE OF Y CHROMOSOME IN THE COW

SHAHARUM BIN SHAMSUDDIN

FSAS 1996 16

THE USE OF ZINC FINGER PROTEIN GENE TO DETECT THE PRESENCE OF Y CHROMOSOME IN THE COW

SHAHARUM BIN SHAMSUDDIN

MAY 1996

MASTER OF SCIENCE

FACULTY OF SCIENCE AND ENVIROMENTAL STUDIES
UNIVERSITI PERTANIAN MALAYSIA.

THE USE OF ZINC FINGER PROTEIN GENE TO DETECT THE PRESENCE OF Y CHROMOSOME IN THE COW

By

SHAHARUM BIN SHAMSUDDIN

Thesis submitted in fulfilment of the requirement for the Degree of Master of Science in Faculty of Science and Enviromental Studies, Universiti Pertanian Malaysia.

May 1996

``Janganlah kamu berhati lemah dalam mengejar mereka. Jika kamu menderita kesakitan, maka sesungguhnya mereka pun menderita kesakitan juga, sebagaimana kamu menderitainya, sedang kamu mengharap daripada Allah apa yang mereka tidak harapkan. Dan adalah Allah itu Maha mengetahui lagi Maha bijaksana ''

An-Nisaa' 104

IN THE NAME OF ALLAH, MOST GRACIOUS, MOST MERCIFUL

Dedicated to :

My Parents : Shamsuddin Hassan and Sharifah Mohamad
My wife : Junaidah Bahran
My children : Asma' Firzanah & Muaz Haqim Shaharum
My Brothers and Sisters
My teachers and research collegues .....

ACKNOWLEDGEMENTS

All praise be to ALLAH, the Almighty, the Merciful and the Beneficent. Had it not been due to His will and favour, the completion of this study would not have been possible.

I wish to express my deep appreciation and gratitude to the committe members, Associate Professor Dr. Abdullah Sipat (Chairman), Associate Professor Dr. Khatijah Mohd. Yusoff, Associate Professor Dr. Mohd. Nazalan Mohd. Najimudin (USM) and Associate Professor Dr. Mohd. Ariff Syed for their constant support and encouragement throughout the course of this study.

I also wish to express my sincere appreciation to the Director General of the Department of Veterinary Services, Malaysia (DVS), Dato' Dr. (Hj.) Hadi bin Dato' Hashim for the financial support on the development of the molecular and celullar biology laboratory. Special thanks to the Director of Planning, DVS, Dr. Hawari Hussein and the Director of Human Resource Development, DVS, Dr. Matta Abdul Rahman for the initiation of the research project and the constant encouragement.

I am grateful to all staff members of the Reproduction Biotechnology Division, Institut Bioteknologi Haiwan Kebangsaan, Jerantut, especially to Dr. Vincent Ng In Hooi (female section) and Dr. V. Krishnalingam (male section) for the helping hand on the clinical and post mortem work. Thanks are also due to the technical assistance provided by En. Mohd. Razli Abdullah and all the Farm's Division staff, IBHK, for the animal husbandry expertise.

Finally I am grateful to all my collegues in the 202 lab. ; Mokhtar, Norwati, Sudani, Haizan, Yow Geok Yong, Nasaruddin, Awang and those who had given their valuable support and helping hands that made this study a successful one. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURE

LIST OF PLATES

LIST OF ABBREVIATIONS

The following abbreviations were used in the text :

EDTA	ethylenediamine tetra acetic acid
DNA	deoxyribonucleic acid
bp	base pair
mg	milligram
µL	microlitre
mL	millilitre
min	minute
mM	millimolar
M	Molar
NBT	nitroblue tetrazolium
X-Phosphate	5-bromo-4-chloro-3-indolyl phosphate
DIG	digoxygenin
UV	ultraviolet
^{0}C	degrees centrigrade
w/v	weight per volume
v/v	volume per volume
ng	nanogram

μg	microgram
χ^2	chi square
ZFX	zinc finger protein gene (X chromosome derived)
ZFY	zinc finger protein gene (Y chromosome derived)
TDF	Testis determining factor
TFM	mouse testicular feminization gene
H_0	Null hypothesis
SRY	Sex determining region Y
Tdy	Mouse testis determining factor
Mafriwal	Malaysian-Freisien-Sahiwal
g	gravity
g	gram
h	hour
ddH_2O	double distilled water
RFLP	Restriction Fragments Length Polymorphism

Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirement for the degree of Master of Science.

THE USE OF ZINC FINGER PROTEIN GENE TO DETECT FOR THE PRESENCE OF Y CHROMOSOME IN THE COW

By

SHAHARUM SHAMSUDDIN
May 1996

Chairman : Associate Professor Abdullah Sipat PhD
Faculty : Science and Enviromental Studies

Total genomic DNA from 24 normal cows and a normal male cattle were isolated from leucocyte preparation. Polymerase Chain Reaction (PCR) amplification of these genomic DNA using the P1-5EZ and P2-3EZ primers for ZFX/ZFY gene resulted in a fragment of approximately 440 bp in size from both male and female samples. This PCR product represents part of the ZFX/ZFY gene defined by the primers used. The PCR products were proven to be amplified from bovine genomic DNA from the dot blot hybridization studies. After *Pst* I digestion, the female samples (ZFX) remained undigested while the male (ZFX/ZFY) showed three fragments of approximately 440 bp, 340 bp and 100 bp. Thus the ZFY gene has a unique *Pst* I site. The PCR product from a

population of 45 female and 47 male cattle was further tested for the presence of the *Pst* I cleavage. There was no *Pst* I site in all the female (ZFX) samples, while 45 out of 47 samples from the male were cleaved. From χ^2 analysis of the data, the *Pst* I cleavage on ZFY was significantly sex dependent. The restriction analysis of ZFX/ZFY gene showed the existence of a unique *Mnl* I site on ZFX and more than one *Alu* I restriction site on both ZFX and ZFY. The PCR product from an infertile female cattle (supposedly, 60, XX and therefore ZFX/ZFX) was subjected to the *Pst* I digestion, and found to be cleaved indicating the presence of the ZFY gene and possibly the Y chromosome. The latter was confirmed by cytogenetic analysis. Post mortem and autopsy evaluation of the animal showed a partial development of male characteristic and a defective female reproduction system. The use of the PCR diagnosis for the presence of the Y chromosome was applied to a population of 30 suspected infertile female animals, and two were found to have the ZFY gene indicating the possible presence of the Y chromosome. Thus, PCR amplification of the ZFX/ZFY gene followed by *Pst* I digestion and analysis on gel electrophoresis can be used as an approach to establish the presence of the Y chromosome in an infertile female cattle. This approach however, needs to be supported by karyotyping to establish unequivocally the screening procedure.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi syarat untuk mendapatkan Ijazah Master Sains.

PENGGUNAAN GEN `ZINC FINGER PROTEIN' UNTUK MENGESAN DEFEKSI GENETIK BERKAITAN KROMOSOM Y PADA LEMBU BETINA

OLEH

SHAHARUM BIN SHAMSUDDIN
Mac 1996

Pengerusi : Profesor Madya Abdullah Sipat, PhD
Fakulti : Sains dan Pengajian Alam Sekitar

DNA genomik dipencilkan daripada persediaan leukosit dari sejumlah 24 ekor lembu betina normal dan seekor lembu jantan. Amplifikasi secara tindakbalas rantai polimerase (PCR) terhadap kedua-dua DNA genomik jantan dan betina tersebut menggunakan primer-primer spesifik gen ZFX/ZFY, P1-5EZ dan P2-3EZ, telah menghasilkan *fragmen-fragmen teramplifikasi bersaiz* kira-kira 440 bp. Produk PCR tadi dibuktikan berasal dari DNA genomik lembu melalui teknik penghibridan dot blot. Selepas pencernaan oleh enzim *Pst* I, sampel betina (ZFX) didapati tidak tercerna manakala sampel jantan menunjukan kehadiran tiga fragmen bersaiz 440 bp, 340 bp dan 100 bp. Ini menunjukan bahawa ZFY mempunyai satu tapak unik *Pst* I. Sejumlah persampelan yang lebih besar, iaitu 45 ekor lembu betina dan 47 ekor lembu jantan, telah digunakan untuk menguji kebolehan pembelahan oleh *Pst* I. Hasilnya tidak ada pembelahan oleh *Pst* I keatas semua sampel betina manakala dua ekor hasil dari lembu

jantan tidak menunjukan pembelahan. Keputusan ujian χ^2 ke atas hasil persampelan tadi menunjukan signifikan pembelahan *Pst* I ke atas gen ZFY adalah bergantung kepada jantina jantan. Dari analisis enzim penyekatan ke atas gen ZFX/ZFY menunjukan kehadiran satu tapak unik *Mnl* I di atas gen ZFX dan lebih daripada satu tapak *Alu* I di atas ke dua-dua gen ZFX dan ZFY. Seekor lembu betina mandul (seharusnya 60, XX dan tentunya terdiri dari ZFX/ZFX) telah diuji terhadap kehadiran ZFX/ZFY dan hasilnya terbukti ia mengandungi gen ZFY seterusnya menunjukan lembu tersebut mengidap defeksi genetik berkaitan kromosom Y. Keputusan ini juga dibuktikan secara analisis sitogenetik. Lembu tersebut seterusnya menjalani bedah siasat dan hasil autopsi didapati ianya menunjukan perkembangan separa struktur pembiakan pejantan disamping sistem pembiakan betina yang cacat. Pendekatan ini seterusnya dilaksanakan ke atas 30 ekor lembu betina bermasalah kesuburan dan di dapati dua ekor daripadanya mempunyai gen ZFY yang menunjukannya mengidapi defeksi genetik berkaitan kromosom Y. Oleh yang demikian, amplifikasi PCR menggunakan gen ZFX/ZFY diikuti pembelahan oleh enzim *Pst* I serta pengamatan gel elektroforesis boleh digunakan sebagai kaedah untuk mengenalpasti kehadiran kromosom Y di dalam lembu betina mandul. Walau bagaimana pun, analisis sitogenetik juga diperlukan bagi mengelakan cerapan penskrenan yang silap.

CHAPTER I

INTRODUCTION

The Y chromosome determines the maleness in mammals, and the initiation of male development in mammals may require one or more genes on the Y chromosome. A Y chromosome linked gene divert the undifferentiated embryonic gonad from the default ovarian pathway to favour testis differentiation, thus initiating male development. In the absence of this gene(s), the gonad develops into the female reproductive system. There have been several genes reported to be the `testis determining factor genes in mammals. Among them are the ZFY/ZFX loci in bovine (Aasen and Medrano, 1990; Pollevick *et al.,* 1992), SRY gene in mice (Koopman *et al.*, 1991), the X-Y homologous primer (Nakahori *et al.*, 1991), the Y-specific repeat sequence in human DYZ1 locus (Handyside *et al.,* 1989), the btDYZ1 in human (Bred backa *et al.*, 1995) and the testis determining factor (TDF) gene localized to Yp11.2-Ypter on the short arm of the human Y chromosome (Hamdorf and Gregg, 1994).

Although the `zinc finger protein' gene was suspected to be a testis determining factor gene for several reasons (Page *et al.,* 1987), a similar gene ZFX, has also been found on the X chromosome of human and mouse. This ZFX gene encodes for the zinc finger protein and it cross-hybridizes to ZFY probes under stringent condition (Palmer *et al.*, 1989). It thus appears that the role of ZFY gene in determining the male sex for human and mouse is not clear cut. Although, both genes appear to be homologous, the ZFY gene was shown to have a unique *Pst* I restriction site which is not present in the ZFX homolog (Aasen and Medrano, 1990). This feature enables one to distinguish ZFY from ZFX and thus to use it to indicate the presence of the Y chromosome for sex determination.

The usage of the gene for sex determination in bovine has been reported in various laboratories through out the world (Epensperger *et al.,* 1989, Aasen and Medrano, 1990, Pollevick *et al.*, 1992, Schellander *et al.,* 1992, Bredbacka *et al.*, 1995 and Peippo *et al.*, 1995). While the ZFX/ZFY gene is useful for sex determination in bovine, its usage can be further extended to screen for chromosomeY-related genetic defect in female animals such as Freemartinism (Schellander *et al.,* 1992) and XXY trisomy (Sysa and Slota, 1984) where the female animal has a Y chromosome in its chromosome make up.

Other than the usage of ZFX/ZFY as a genetic marker for sexing and for screening for the `Y-related genetic defects' (Schellander *et al.*, 1992), the ZFX/ZFY gene can also be used to determine the probable cause of infertility problems among the elite cows. A large amount of the ZFX/ZFY DNA can be obtained by amplification using the polymerase chain reaction (PCR) followed by *Pst* I cleavage analysis. This thesis is about the application of ZFX/ZFY loci as a method for the screening of chromosome-Y related genetic defect in infertile cows.

Objectives

The objective of this study is to amplify the zinc finger protein gene from bovine genomic DNA derived from leucocytes by PCR. The primers involved can then be used as a specific marker for sex determination in cattle as well as a precise diagnostic tool for rapid detection of `Y related genetic defect' among female cattles during the breeding programme.

Since the elite cows play an important role in the Department of Veterinary Services (DVS) breeding programme, especially in artificial insemination (AI) and multiple ovulation embryo tranfer (MOET). The development of a precise and efficient detection of such genetic abnormalities could give a great economical impact in terms of saving costs for animal husbandry and also the improvement of fertility performance of the animals.

CHAPTER II

LITERATURE REVIEW

Chromosome composition of cattle.

There are two types of chromosomes in a mammalian cell, *viz.* the autosomes (non-sex chromosomes) and the sex chromosomes (X and Y). The male animal has the XY chromosome composition while the female animal has the XX type. Different species of mammals show varying chromosome numbers. In cattle the diploid number of a normal female is 2n = 60, XX while the normal male is 2n = 60, XY. All the autosomal chromosomes in bovine except for the sex chromosomes are structurally acrocentric ; v-shape with the centromere at the apex. In *Bos indicus* and *Bos taur-indicus* crossbreds, the Y chromosome is also structurally acrocentric (Y `v') but is much smaller and thus, can be confused with the other autosomes. In pure bred *Bos taurus*, the Y chromosome is a small metacentric (Y `x') chromosome (Halnan, 1989).

It has been proposed that the difference between the Y chromosomes of *Bos taurus* and *Bos indicus* is due to a `balanced translocation' among the Y chromosome and an autosome (Rendel, 1980). However, there is no distinct differentiation among the X chromosome in any breed.

Fortunately, the differences in the structure of Y chromosomes do not present a consistent relationship to infertility (Halnan, 1989). If the Y chromosome gives some clues to the cause of infertility, the X chromosome also might be informative. However the data relating to the X chromosome is not much reported although there are variations in size between the X chromosomes in each cell of a given cow in several breeds (Halnan, 1989).

The role of Y chromosome in sex determination.

The Y chromosome is required for normal male development in mammals. In addition to carrying genes controlling sex determination and perhaps spermatogenesis, the Y chromosome must also pair with the X chromosome during the meiosis process. The Y chromosome varies in size and in *Bos taurus* there is no relationship between the size of the Y chromosome and infertility (Halnan, 1989).

The relationship between structure and function for the Y chromosome can be studied by molecular cloning and analysis of Y derived sequences. Although in cattle, the X and Y chromosomes are different in structural appearance and genetic content (Halnan, 1989), they are believed to be derived from an ancestral pair of chromosomes differing at only a single, sex determining locus (Ohno, 1967). Thus the homology of the gene(s) should be possible and indeed many of the unique sequences cloned from the Y chromosome hybridized to the X chromosome as well (Page *et al.*, 1983 ; Schneider *et al.*, 1989).

Furthermore, several clones isolated from random genomic libraries, and from X chromosome enriched libraries, were found to share sequences with the Y chromosome, for example, the zinc finger protein gene (Page *et al.*, 1982).

From immunogenetics studies, the autosomes as well as the sex chromosomes play the role in sex determination (Halnan, 1989). Up to the time of the discovery of H-Y antigen (Wachtel, 1977), genetic mechanisms for sex differentiation and fertility were attributed to specific loci on sex chromosomes.

Sharp *et al.* (1980) focused attention on the male determining role of HY antigen. The H-Y antigen is a component of the plasma membrane acting in conjunction with gonad specific receptor anchorage site B2-MHC (Ohno *et al.*, 1979) which is integral to the differentiation of the undifferentiated gonad to a testis (Halnan, 1989). Expression of H-Y antigen is attributed to a locus near the centromere of the Y chromosome in mammals (Wachtel, 1977). This expression is due to specific loci on the Y, the X and at least one autosome chromosome in mammals (Wachtel, 1977). However further maturation of sex is not H-Y dependent, other genes that operate include the testicular feminization gene (TFM), the locus for which is on the X chromosome. These systems and their genes operate in the presence of genes for binding sites on the cell plasma or in the cytoplasm (Halnan, 1989).

Abnormal chromosome composition in cattle

Chromosomal aberrations do occur in cattle and they may cause reproductive problems. The most common chromosomal abnormalities in cattle reported are the XX:XY chimaeras (Freemartinism), trisomy X (XXX), sex chromosome mosaics, autosomes variants (61XX or XY), polyploidy, the Robertsonian 1/29 translocation and tandem fusion translocation (Halnan, 1989).

Sex chromosome anomalies in cattle are usually associated with infertility and of these, Freemartinism is the most commonly reported (Halnan, 1989). The phenomenon of Freemartinism has been known to occur in cattle since ancient times (Marcum, 1974) in the heterosexual twin female. The promotion of twins in commercial animal production has resulted in an increase of cases of Freemartinism in heifers that are born twin to a bull. Freemartinism arise as a result of shared placental circulation of heterozygous foetuses in multiple pregnancies involving male and female foetuses (Lillie, 1965).

Many of these animals, especially from dairy farms, were grown and treated exclusively without knowing that they are Freemartins. Generally, 92% of heifers born co-twin to bulls are infertile (Marcum, 1974). Therefore, there is a significant economic importance on an early diagnosis of Freemartinism. Cytogenetically, Freemartinism manifests itself as chimerism of sex chromosome (2n = 60, XX/XY) between cells and it is a standard practice to diagnose Freemartinism by examination of