



Singleton freemartinism in cattle: A structured narrative review and historical case reappraisal

Hiroya Kadokawa 

Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi, 753-8515, Japan

ARTICLE INFO

Keywords:

Cattle
Diagnosis
Fetal loss
Freemartinism
Singleton
XX/XY chimerism

ABSTRACT

Freemartinism classically affects a female calf gestated with a male co-twin. A female may be born as an apparent singleton when a male co-conceptus is lost after placental vascular communication has permitted cellular exchange and exposure to testicular products. This structured narrative review and historical case reappraisal evaluates evidence for singleton freemartinism, clarifies diagnostic boundaries and reassesses a 1995 case following multiple-embryo transfer. PubMed, Scopus and FAO AGRIS were searched through 14 September 2026, supplemented by citation searching. Evidence supports the existence of singleton freemartinism but does not establish its prevalence. An apparent singleton birth does not demonstrate that gestation was singleton throughout. In an appropriate developmental context, persistent XX/XY blood chimerism, the coexistence of XX and XY blood-cell populations, is evidence that fetal cell exchange occurred. Detection of a Y-linked sequence establishes only that target DNA is present in the specimen. Neither finding alone demonstrates male-type reproductive-tract development or pathological freemartinism. Diagnosis therefore requires integration of gestational history, characterized chromosome-based or molecular findings, reproductive anatomy, functional findings and histopathology. Archival sections show ovarian follicles and testis-like tubular structures in the same gonad. Interpretation is limited by the unknown sex of the lost embryo, incomplete gonadal examination, lack of cell-level localization and cell-type identification, and unknown fertility. Twinning, multiple-embryo transfer and conceptus loss may increase opportunities for the condition to arise, but evidence demonstrates neither increased prevalence nor a quantitative contribution to reproductive failure. Prospective multicenter studies linking gestational records with molecular, anatomical and functional outcomes are required.

1. Introduction

Freemartinism is a well-recognized disorder of sexual development in cattle and an important cause of infertility in phenotypic females gestated with a male co-twin. Placental vascular connections (anastomoses) permit cells and signaling molecules to pass between the fetal circulations. This exchange can produce persistent blood-cell chimerism, the coexistence of genetically distinct blood-cell populations, and expose the female fetus to testicular products during susceptible stages of reproductive development [1–4]. The resulting abnormalities vary from subtle changes of the tubular reproductive tract to extensive male-type development of the gonads (gonadal masculinization) and sterility.

The biological mechanism and the evidence required to diagnose an individual animal must nevertheless be distinguished. XX/XY leukocyte chimerism is strongly associated with freemartinism in the appropriate gestational context, but a blood finding alone does not demonstrate that

the reproductive tract or gonads underwent male-type development. Conversely, infertility, repeat breeding or an abnormal reproductive tract has many possible causes and should not be attributed to freemartinism without appropriate investigation. This distinction becomes especially important when a female is born without a male co-twin.

Apparently singleton-born freemartins have been reported under natural breeding and embryo-transfer conditions. Wijeratne et al. [5] described sterile single-born heifers with XX/XY chimerism and reproductive-tract abnormalities, and Murakami et al. [6] subsequently reported a single-birth freemartin identified by chromosome analysis among sterile heifers. In 1995, Kadokawa et al. [7] reported a phenotypic female born as a singleton after multiple embryo transfer in which a male-specific DNA sequence and a testis-like tubular structure in one gonad were detected. These reports support the occurrence of the condition, but their small number, selected clinical populations and differing diagnostic methods preclude estimation of prevalence.

The most plausible mechanism is loss of a male co-conceptus after a

E-mail address: hiroya@yamaguchi-u.ac.jp.

<https://doi.org/10.1016/j.theriogenology.2026.118225>

Received 3 June 2026; Received in revised form 23 September 2026; Accepted 27 September 2026

Available online 1 October 2026

0093-691X/© 2026 The Author. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

functional vascular connection with the surviving female has developed. Under this model, the observed singleton birth is the final pregnancy outcome and does not imply that gestation was singleton throughout. Recent genomic identification of blood chimerism in some cattle registered as singletons is consistent with unrecorded fetal cell exchange [8]. Such findings establish neither the sex and timing of loss of the previous conceptus nor pathological freemartinism in the surviving animal unless reproductive findings are also available.

Changes in dairy-cattle reproduction, including greater opportunity for multiple ovulation, twin gestation and use of assisted reproductive technologies, make the hypothesis relevant to contemporary practice. They do not, however, demonstrate that singleton freemartinism has increased in prevalence. Likewise, the possibility of underdiagnosis should be treated as a testable hypothesis rather than as an established explanation for an appreciable proportion of unexplained infertility.

This article is therefore positioned as a structured narrative review combined with a historical case reappraisal. Its objectives are to synthesize the available evidence for apparently singleton-born freemartins; distinguish gestational opportunity, fetal cell exchange, XX/XY chimerism, reproductive pathology and definitive diagnostic inference; reassess the 1995 multiple-embryo-transfer case using archival histopathological material; compare available diagnostic approaches; and define priorities for future population-based investigation. The historical case is treated as one component of the evidence base and not as a basis for epidemiological or production-level conclusions.

2. Scope and literature-search strategy

2.1. Review design and scope

This article is a structured narrative review combined with a historical case reappraisal. Its principal aims are to: (i) distinguish the established biological mechanism of freemartinism from the evidence required to diagnose the condition in an individual animal; (ii) critically synthesize reports of apparently singleton-born cattle with XX/XY chimerism or freemartin-like reproductive abnormalities; (iii) reassess the previously reported 1995 singleton case in light of previously unpublished histopathological images and subsequent advances in cytogenetic, molecular, endocrine and clinical diagnosis; and (iv) identify testable questions for future population-based research. The review was not designed as a prevalence meta-analysis because the available literature consists of heterogeneous case reports, selected clinical series, experimental studies and studies using different diagnostic definitions.

For the purposes of this review, *singleton-born* or *apparently singleton-born* describes the observed birth outcome and does not establish that only one conceptus was present throughout gestation. *XX/XY chimerism* denotes the presence of XX and XY cell populations in the same animal. *Freemartinism* is used for the pathological syndrome resulting from heterosexual co-gestation and characterized by variable disruption of female reproductive development. These terms are not treated as interchangeable.

2.2. Electronic literature searches

Electronic searches of PubMed, Scopus and the Food and Agriculture Organization of the United Nations International System for Agricultural Science and Technology (FAO AGRIS) were completed on 14 September 2026. No restrictions were imposed on publication year or language. Scopus was searched without document-type or subject-area restrictions. Search concepts covered freemartinism, XX/XY chimerism, leukochimerism, singleton birth, heterosexual multiple gestation, embryo transfer, embryonic or fetal loss, diagnostic methods, reproductive-tract and gonadal development, placental vascular anastomosis, endocrinology, masculinization and virilization.

The PubMed search was deliberately divided into a high-sensitivity principal search and four question-specific supplementary searches

because some relevant articles use *XX/XY chimerism* or *leukochimerism* without using *freemartin* in the title or abstract. The five PubMed searches returned 122, 22, 56, 119 and 105 records, respectively. After deduplication across the five searches, 207 unique PubMed records remained.

The Scopus search of article titles, abstracts and keywords returned 324 records. Comparison with the 207-record PubMed set identified 157 matches: 141 by PubMed identifier, eight by DOI and eight by normalized title. Thus, 167 Scopus records not matched to the PubMed set underwent separate title-and-abstract screening.

The corresponding FAO AGRIS search returned 166 records. AGRIS records supplied by more than one data provider were treated as duplicate bibliographic records rather than separate studies. Because AGRIS searches the metadata supplied by participating institutions and includes gray literature as well as journal articles, AGRIS-only records were treated as candidate sources and were not cited until their bibliographic identity and relevant findings could be verified from an original or authoritative source.

The exact electronic search expressions and the number of records retrieved are presented in Table 1.

2.3. Supplementary discovery and bibliographic verification

The electronic searches were supplemented by backward citation searching of the submitted manuscript, relevant reviews and eligible original articles. Forward citation information in Scopus was used selectively to identify later diagnostic and case reports. Publisher websites and DOI metadata were used to verify titles, author lists, publication years, journal names, volumes, pages or article numbers and DOIs. PubMed records were used to verify PubMed-indexed studies. Historical papers not indexed in PubMed were retained only when the bibliographic identity could be confirmed from the original journal record, an authoritative publisher record or a traceable reference in the historical literature.

2.4. Eligibility and selection of evidence

Titles and available abstracts were screened for relevance. Full texts were examined for retained studies used to support substantive statements whenever accessible. A record for which the relevant findings could not be verified was not used to support a substantive scientific claim, although a bibliographically verified historical record could be cited solely to document the history of the field. The following evidence was eligible for inclusion.

1. Reports of singleton-born or apparently singleton-born cattle or buffaloes with cytogenetic, molecular, anatomical, histopathological, endocrine or reproductive findings relevant to freemartinism;
2. Studies of heterosexual bovine twins or multiple births that directly addressed placental vascular exchange, hematopoietic chimerism, reproductive-tract differentiation, gonadal development, endocrine function or phenotypic variability;
3. Studies evaluating clinical examination, vaginal length, ultrasonography, vaginoscopy, karyotyping, leukocyte culture, blood-group testing, fluorescence in situ hybridization, polymerase chain reaction (PCR)-based assays, sequencing, droplet digital PCR, loop-mediated isothermal amplification, anti-Müllerian hormone measurement or pathological examination for freemartin assessment;
4. Studies of twinning, embryo transfer, spontaneous conceptus loss or twin reduction that informed the biological opportunity for a previous heterosexual multiple gestation; and
5. Reports of other bovine differences of sex development when required for differential diagnosis.

Studies were excluded when freemartinism was mentioned only incidentally; when they concerned unrelated aspects of nutrition,

Table 1
Electronic search strategies and yields.

Database/search	Search expression	Records retrieved
PubMed principal search	(freemartin[Title/Abstract] OR freemartinism[Title/Abstract]) AND (cattle[Title/Abstract] OR bovine [Title/Abstract] OR heifer[Title/Abstract])	122
PubMed terminology search	("XX/XY chimerism"[Title/Abstract] OR "sex-chromosome chimerism"[Title/Abstract] OR leukochimerism[Title/Abstract] OR "blood chimera"[Title/Abstract]) AND (cattle[Title/Abstract] OR bovine[Title/Abstract] OR heifer [Title/Abstract])	22
PubMed singleton and conceptus-loss search	(freemartin[Title/Abstract] OR freemartinism[Title/Abstract] OR "XX/XY chimerism"[Title/Abstract]) AND (singleton[Title/Abstract] OR single-born[Title/Abstract] OR "single birth"[Title/Abstract] OR "co-twin"[Title/Abstract] OR "heterosexual twin"[Title/Abstract] OR "embryo transfer"[Title/Abstract] OR "embryonic loss"[Title/Abstract] OR "fetal loss"[Title/Abstract] OR "foetal loss"[Title/Abstract] OR "vanishing twin"[Title/Abstract] OR "twin reduction"[Title/Abstract])	56
PubMed diagnostic search	(freemartin[Title/Abstract] OR freemartinism[Title/Abstract] OR "XX/XY chimerism"[Title/Abstract]) AND (diagnos*[Title/Abstract] OR karyotyp*[Title/Abstract] OR cytogen*[Title/Abstract] OR PCR[Title/Abstract] OR qPCR[Title/Abstract] OR FISH[Title/Abstract] OR LAMP[Title/Abstract] OR ddPCR[Title/Abstract] OR SRY[Title/Abstract] OR AMELY[Title/Abstract] OR AMH[Title/Abstract] OR ultrason*[Title/Abstract] OR vagin*[Title/Abstract] OR histopath*[Title/Abstract])	119
PubMed developmental and pathological search	(freemartin[Title/Abstract] OR freemartinism[Title/Abstract] OR "XX/XY chimerism"[Title/Abstract]) AND (placent*[Title/Abstract] OR anastomos*[Title/Abstract] OR gonad*[Title/Abstract] OR Mullerian[Title/Abstract] OR "anti-Mullerian"[Title/Abstract] OR steroid*[Title/Abstract] OR endocrin*[Title/Abstract] OR masculin*[Title/Abstract] OR viriliz*[Title/Abstract])	105
Scopus	(freemartin* OR "XX/XY chimerism" OR leukochimerism) AND (cattle OR bovine OR heifer* OR cow OR calf OR calves) searched in article titles, abstracts and keywords	324
FAO AGRIS	(freemartin* OR "XX/XY chimerism" OR leukochimerism) AND (cattle OR bovine OR heifer* OR cow OR calf OR calves) searched anywhere in the bibliographic record	166

behavior, production or transplantation; when they described non-bovine species without contributing directly to a relevant mechanism; or when the source could not be identified sufficiently to verify its bibliographic details or findings. Duplicate database records and duplicate reports of the same animals were counted once unless a later publication contributed distinct anatomical, molecular or pathological evidence.

Studies of increased twinning, embryo transfer or conceptus loss were used only to evaluate whether the opportunity for a transient male co-conceptus may have increased. They were not interpreted as

evidence that the prevalence of singleton freemartinism has increased. Similarly, studies of infertile or repeat-breeding heifers were not treated as evidence of singleton freemartinism unless the animals underwent appropriate diagnostic investigation.

2.5. Evidence framework for apparently singleton-born animals

To avoid equating fetal cell exchange with pathological freemartinism, evidence from each apparently singleton-born animal or selected case series was separated into the following domains.

- 1. Gestational evidence:** documented singleton gestation, apparently singleton birth, embryo transfer, known multiple gestation, observed co-twin loss or unknown gestational history.
- 2. Cellular or molecular evidence:** XX/XY leukocyte chimerism, detection and quantity of Y-linked sequences, tissue distribution of XY cells and the analytical method used.
- 3. Phenotypic and anatomical evidence:** external genital phenotype, vaginal length, cervical and uterine development, ultrasonographic or post-mortem findings and gonadal morphology.
- 4. Histopathological evidence:** ovarian tissue, testicular or seminiferous structures, mixed ovarian and testicular differentiation and reproductive-tract histology.
- 5. Functional evidence:** cyclicity, endocrine findings, mating or insemination outcome and confirmed fertility or sterility.

Detection of XY leukocytes or a Y-linked sequence in blood was interpreted as evidence of male-derived cellular material and therefore of fetal cell exchange or another source of chimerism. It was not considered, by itself, definitive evidence of reproductive-tract masculinization, gonadal transformation or pathological freemartinism. Conversely, infertility or a reproductive-tract abnormality without evidence of heterosexual cell exchange was not assumed to represent freemartinism. Conclusions for individual reports were calibrated to the combination of evidence domains actually examined.

2.6. Approach to synthesis

The eligible literature was synthesized narratively because study designs, animal selection, diagnostic definitions, tissues examined and outcome measures were too heterogeneous for quantitative pooling. Historical observations and the 1995 case were integrated with, but not given greater evidential weight than, subsequent cytogenetic, molecular, endocrine, pathological and population-level studies. The previously unpublished histopathological images from the 1995 case were used to clarify the morphology of the recovered gonad; they were not treated as new prevalence data. The failure to recover the contralateral gonad is considered explicitly as a limitation of that case.

3. Biological basis of freemartinism and levels of evidence

3.1. Placental vascular communication and hematopoietic chimerism

In its classical form, bovine freemartinism follows a heterosexual multiple pregnancy in which vascular connections develop between the fetal placental circulations. Lillie [1] proposed that these connections permit substances from the male fetus to alter sexual development in its female co-twin. Owen [2] subsequently demonstrated long-lasting mixtures of red-blood-cell types in dizygotic cattle twins, establishing that placental vascular communication also permits the exchange and persistence of hematopoietic cells. These observations underpin two related but distinct consequences of a shared circulation: stable blood-cell chimerism and disruption of reproductive development in the female fetus [4].

The cellular consequence has been demonstrated directly. In a 60-day freemartin fetus, Niku et al. [9] identified Y-chromosome-positive,

CD34-positive hematopoietic progenitors (immature blood-forming cells) in the fetal liver, together with pronounced chimerism in the thymus. Exchanged male-derived progenitors can therefore colonize hematopoietic tissues and contribute to a persistent XX/XY leukocyte population. The proportion of XY cells measured after birth reflects the surviving hematopoietic lineages in the sampled tissue, however, and should not be interpreted as a direct measure of either the amount of endocrine exposure or the severity of male-type reproductive-tract development. Chimerism may also differ among tissues; consequently, a result obtained from blood does not by itself establish the chromosomal composition or differentiation of a gonad.

3.2. Disruption of female reproductive development

Reproductive abnormalities arise when vascular communication exposes the female fetus to testicular products during susceptible stages of sexual differentiation. Anti-Müllerian hormone (AMH), produced by fetal Sertoli cells, promotes regression of the Müllerian ducts, the embryonic precursors of the uterus, oviducts and upper vagina. In heterosexual bovine twins examined between 50 and 80 days of gestation, Müllerian duct regression occurred concurrently in males and freemartins, and serum AMH concentrations were high and positively correlated between the co-twins. By contrast, AMH production by the freemartin gonads themselves was detectable at a low level in only two of 13 fetuses during this period. These findings indicate that early Müllerian regression in the freemartin is mediated principally by AMH originating from the male co-twin rather than by the initially undifferentiated female gonad [10].

Gonadal differentiation and steroid hormone production may also be altered. Freemartin ovaries collected at 47–130 days of gestation produced more testosterone than age-comparable normal fetal ovaries, while estrogen production was undetectable in the younger freemartin gonads examined. Thus, abnormal steroid-producing cell differentiation can be detected from approximately day 47 of gestation [11]. Testicular differentiation within an affected XX gonad can occasionally be extensive. Harikae et al. [12] described seminiferous-tubule-like structures containing Sertoli-like cells positive for SOX9, a marker of testicular supporting-cell differentiation, together with steroid-producing Leydig-like cells and peritubular myoid cells. Such marked gonadal sex reversal demonstrates the possible severity of the developmental response, but it should not be regarded as the uniform or defining morphology of all freemartins.

The resulting phenotype is heterogeneous. Reported abnormalities include a shortened vagina, an enlarged clitoris, hypoplasia or segmental absence of the cervix, uterus and uterine tubes, and gonads ranging from underdeveloped ovarian structures to testis-like tissue or an ovotestis, a gonad containing both ovarian and testicular tissue [4]. Variation probably reflects several interacting factors, including when vascular communication became functional, the developmental interval during which it persisted, and variation in the exposure and responsiveness of individual tissues to male-derived signals. The available evidence does not support prediction of anatomical severity from the postnatal proportion of XY-positive blood cells alone.

3.3. Blood chimerism is evidence of fetal cell exchange, not a diagnosis by itself

For the purposes of this review, five questions must be considered separately: (i) whether the gestational history provides an opportunity for exchange, for example a documented heterosexual co-twin or multiple-embryo transfer; (ii) whether there is evidence that fetal cell exchange occurred; (iii) whether a test demonstrates true XX/XY cellular chimerism in a specified tissue, rather than only the presence of a Y-chromosome sequence; (iv) whether anatomical, endocrine, histopathological or functional findings demonstrate abnormal female reproductive development; and (v) whether the combined evidence supports

a diagnosis of freemartinism. These questions are related, but none is interchangeable with the others. Histopathological demonstration of testicular differentiation within an abnormal gonad provides particularly strong evidence of the reproductive phenotype, but it does not, by itself, reconstruct the preceding pregnancy.

The distinction is supported experimentally. Vigier et al. [13] surgically interrupted vascular anastomoses between bovine conceptuses on days 37 or 45. Three female fetuses subsequently lacked the initial gonadal and Müllerian manifestations of freemartinism despite substantial XX/XY chimerism in the liver. Thus, transferred XY hematopoietic cells can persist without being sufficient to cause the characteristic reproductive pathology. Conversely, a molecular test performed on blood is designed primarily to detect male-derived cells or DNA; it does not directly examine the vagina, Müllerian derivatives or gonads. A positive blood result should therefore be described as evidence of XX/XY blood chimerism and interpreted alongside reproductive anatomy and, where relevant, endocrine, imaging, functional or histopathological findings. This terminology is particularly important when screening females recorded as singletons, because the prior presence, sex and time of loss of another conceptus are usually unknown.

A diagnosis of pathological freemartinism should consequently rest primarily on evidence of disordered female reproductive development in an appropriate biological context. Demonstration of XX/XY chimerism can strongly support that diagnosis but cannot replace examination of the reproductive phenotype. Histopathology can establish the nature of an abnormal gonad, including an ovotestis, but is generally available only after gonadectomy or death and is not required in every clinically recognizable case. Where neither a compatible gestational history nor chimerism is demonstrated, masculinization or gonadal abnormality should also prompt consideration of other differences of sex development. The relative roles and limitations of karyotyping, fluorescence in situ hybridization, PCR-based detection, AMH measurement, ultrasonography, vaginoscopy and histopathology are compared in the diagnostic section of this review.

3.4. Biological plausibility of an apparently singleton freemartin

The classical mechanism does not require the male co-twin to survive until birth. If a male conceptus shares a functional placental circulation with a female fetus and is subsequently lost, the surviving female may be born alone while retaining male-derived hematopoietic cells and, if developmental exposure was sufficient, abnormalities of the reproductive tract or gonad. This sequence provides a biologically plausible mechanism for singleton freemartinism. Nevertheless, apparent singleton birth alone does not demonstrate that an unobserved male co-twin existed, and blood chimerism alone does not demonstrate that masculinization occurred. Evidence of a previous heterosexual conceptus may be strengthened by early ultrasonographic records, embryo-transfer records, examination of fetal or placental remnants, or other reliable reproductive records; in most naturally conceived historical cases, such evidence is unavailable.

Accordingly, the term *singleton freemartin* is used here for a phenotypic female born without a male co-twin at parturition in which reproductive developmental abnormalities compatible with freemartinism are demonstrated and the proposed vanished-male mechanism is supported by the available gestational, cytogenetic or molecular evidence. The strength of that classification depends on the concordance and completeness of the evidence in an individual report. Animals recorded as singletons in which only XX/XY blood chimerism or a Y-chromosome marker has been detected are considered *chimeric singleton females* unless accompanying phenotypic or pathological evidence establishes freemartinism. This operational distinction prevents estimates of blood chimerism from being treated as estimates of singleton freemartin prevalence and provides the framework for evaluating the historical and contemporary reports in the following sections.

4. Historical and contemporary evidence for singleton freemartinism

Reports concerning females born without a male co-twin vary substantially in evidential strength. Some combine a recorded singleton birth, XX/XY leukocyte chimerism and abnormalities of the reproductive tract or gonad; others demonstrate only male-derived cells or DNA in blood. In accordance with the definitions established in Section 3, the latter observations are evidence of fetal cell exchange in an animal recorded as a singleton, but are not by themselves evidence of pathological freemartinism. This distinction is essential when interpreting the small and heterogeneous literature summarized below.

4.1. Early clinical and cytogenetic reports

Wijeratne et al. [5] provided the earliest case series specifically reported as single-birth freemartinism. Among 34 heifers investigated after failure to conceive to three or more inseminations or natural services, 12 had sex-chromosome chimerism and five of these were single-born. Post-mortem examination was performed in three singleton chimeras. Two Friesian heifers had exhibited normal estrous cycles and appeared normal on clinical reproductive examination, but one had a non-patent cervix and the other had non-patent uterine horns; both had functional ovarian structures. The third heifer had a normal vagina, an enlarged clitoris, seminal vesicles and gonads described as resembling fetal testes with primitive ovarian and testicular structures. The two surviving singleton chimeras had normal external genitalia but rudimentary uterine horns and no palpable gonads. The concordance of an apparent singleton history, leukocyte chimerism, infertility and reproductive abnormalities makes this series important evidence that the freemartin phenotype can occur when no male co-twin is present at birth. Its interpretation is nevertheless constrained by the small, clinically selected sample, the limited description of how singleton status was established and the absence of published histopathological images of the gonads. The report therefore demonstrates existence, not frequency.

Murakami et al. [6] subsequently performed cytogenetic investigations in 18 sterile heifers and identified three different chromosomal abnormalities: one 60,XY female, one 60,XX/60,XY single-birth freemartin and one heifer with 61,XX,+20 trisomy; the remaining heifers had a normal karyotype. This study is valuable for two reasons. First, it independently documented XX/XY chimerism in a sterile heifer recorded as a single birth. Secondly, the occurrence of distinct cytogenetic diagnoses within the same clinical series illustrates that sterility in a phenotypic female is not specific for freemartinism. The singleton history and leukocyte karyotype supported the authors' classification, but one animal cannot inform population prevalence, and the report should not be taken to imply that all sterile single-born heifers with a sex-chromosome abnormality share the same developmental mechanism.

Together, these early reports established that pathological freemartinism in an apparent singleton is biologically and clinically credible. They did not establish how often it occurs, how reliably an earlier male conceptus can be reconstructed from postnatal findings, or whether mildly affected animals remain undetected.

4.2. Single-born heifers selected for reproductive-tract abnormalities

Szczerbal et al. [14] examined 12 single-born heifers that had been culled because of underdevelopment of the internal reproductive tract; all had externally female genitalia. Conventional cytogenetic examination of peripheral blood identified 60,XX/60,XY leukocyte chimerism in seven animals, with the proportion of XY metaphases ranging from 8% to 64%. Polymerase chain reaction (PCR) detected two Y-linked sequences in the same seven heifers: SRY (sex-determining region Y) and AMELY (Y-linked amelogenin). The other five animals had a 60,XX

karyotype and no detectable Y-linked sequences, indicating that reproductive-tract underdevelopment within this selected group was not attributable uniformly to freemartinism. Gonadal histopathology was not reported.

This series strengthens the evidence that some single-born heifers with compatible reproductive abnormalities are blood chimeras. It also shows the diagnostic value of combining phenotype, cytogenetics and molecular testing rather than assigning causation from infertility or anatomy alone. However, the study was not a survey of unselected singleton females: entry required reproductive-tract underdevelopment and culling, eight of the 12 heifers came from one dairy breeding center, and the number of all eligible singleton females was not supplied. The observation that seven of 12 selected heifers were chimeric therefore cannot be used as an estimate of singleton freemartin prevalence.

Farm records from the principal breeding center showed contemporaneous increases in milk production and twinning, and Szczerbal et al. [14] proposed that loss of one fetus from a multiple pregnancy could account for some chimeric singletons. These data are compatible with the vanished-co-twin mechanism, but they are ecological observations from a single center and do not demonstrate that increasing milk yield or twinning caused an increase in singleton freemartinism. They indicate an opportunity for the condition to occur and justify targeted investigation; they do not establish a temporal change in prevalence.

4.3. A singleton following multiple-embryo transfer

Kadokawa et al. [7] investigated 22 singleton female calves produced after transfer of multiple, non-sexed embryos. Twenty-one showed neither cytogenetic nor PCR evidence of Y-chromosome material. One phenotypic female was positive for male-specific DNA in blood by PCR despite a normal female result on the cytogenetic examination performed, a combination consistent with a male-derived blood-cell population below the detection threshold of the chromosome count. The external and internal genitalia were reported to be grossly normal in form and size, but a very small seminiferous-tubule-like structure was observed within the examined left gonad. Embryo-transfer records made an earlier male conceptus more directly plausible than in naturally conceived cases for which the pregnancy history was reconstructed only after birth.

The case is informative because it brings together the transfer of multiple non-sexed embryos, a male-specific molecular signal and focal testicular differentiation within an otherwise ovarian gonad. It remains a single observation and therefore provides no basis for estimating epidemiology or contemporary herd-level impact. Moreover, only one gonad was available for evaluation; the contralateral gonad could not be recovered at the slaughterhouse. The distribution and extent of gonadal differentiation in the animal as a whole are consequently unknown. Section 5 reappraises this case and presents the archival gross and histopathological material, including this limitation, in detail.

4.4. Evidence from planned embryo reduction

The vanished-co-twin mechanism does not imply that every female surviving the loss of a male conceptus becomes a freemartin. Garcia-Ispuerto et al. [15] followed 95 single-born females that remained after manual reduction of twin or triplet pregnancies at 28–34 days of gestation and compared their subsequent reproductive performance with that of 1688 control heifers. Pregnancy by 15 months, culling for infertility and calving before 24 months did not differ between groups. The sex of each reduced conceptus was not established and the surviving heifers were not reported to have undergone cytogenetic or molecular chimerism testing; the inference that approximately half had previously had a male co-conceptus was probabilistic. Nevertheless, this controlled field observation is an important counterweight to simple risk extrapolation: reduction during this interval did not measurably impair the reported reproductive outcomes of the surviving females. It does not

determine whether vascular communication had occurred in individual pregnancies.

4.5. Genomic and molecular observations in animals recorded as singletons

Population-scale genomic data have added evidence that fetal blood-cell exchange may be detected in cattle recorded as singletons. Lindtke et al. [8] analyzed low-pass sequencing data from blood samples of 322 beef-dairy F1 cattle and identified 20 putative blood chimeras from increased genome-wide heterozygosity. Fifteen of 18 animals reported as twins showed this signal, as did five animals reported as singletons. Hair-bulb single-nucleotide polymorphism (SNP) array data from the same animals did not show corresponding chimerism, supporting restriction of the discordant cell populations to blood rather than the germline tissue sampled. These results provide a scalable means of recognizing blood chimerism and support the possibility that an unrecorded or lost co-twin may precede some singleton births. The study did not report reproductive-tract phenotypes, gonadal histopathology or fertility outcomes for the five alleged singletons. They should therefore be classified as putative blood-chimeric singleton cattle, not as five confirmed singleton freemartins. The authors' use of the term "alleged singletons" also appropriately recognizes that routine birth records may not reconstruct early gestation completely.

Maylem et al. [16] extended molecular detection to cattle and water buffalo by comparing qualitative PCR and quantitative PCR assays targeting *SRY*, alongside amelogenin amplification and recorded morphology. Their series included one water buffalo recorded as a singleton with deformed external genitalia and detectable *SRY* and X- and Y-associated amelogenin products. This observation provides a valuable recent example of how molecular testing can identify male-derived genetic material in a singleton with an external phenotype compatible with masculinization. The study also usefully illustrates the range of external and internal morphologies encountered among suspected or clinically designated freemartins and the potential of quantitative assays to estimate the relative abundance of an *SRY* signal.

Maylem et al. provide valuable molecular and morphological information for interpreting suspected freemartinism. For the singleton water buffalo, the reported evidence comprised external genital deformation together with the *SRY* and amelogenin findings. The underdeveloped uterine tract described elsewhere in the study concerned another animal, whereas detailed examination of the internal reproductive tract, cytogenetic confirmation and histopathological assessment were not reported for the singleton. On the evidence available, it can be described as an *SRY*-positive singleton water buffalo with an external phenotype compatible with suspected freemartinism. Because the present review concerns cattle, this observation is included as supportive cross-species evidence and as a useful example of integrating molecular and phenotypic findings.

4.6. Critical synthesis of the evidence

The available reports support three conclusions. First, they support the existence of singleton freemartinism: apparent singleton birth, XX/XY blood chimerism and compatible reproductive abnormalities occurred together in the clinical series of Wijeratne et al. [5] and Szczerbal et al. [14], while the embryo-transfer case of Kadokawa et al. [7] combined molecular evidence with focal gonadal differentiation. Secondly, evidential completeness is uneven across reports. Gestational history is often retrospective, reproductive anatomy is described incompletely, histopathology is rarely available, and molecular assays do not all answer the same question. Thirdly, neither the historical case series nor modern genomic screening provides a denominator that permits estimation of the prevalence of pathological singleton freemartinism.

Table 2 separates what each principal report demonstrated from what it could not establish. This prevents a molecular signal in blood from being counted as a reproductive disorder and prevents a clinically selected series from being treated as a population survey. On present evidence, it is reasonable to hypothesize that some singleton freemartins are not recognized by birth history alone and to test this hypothesis prospectively. It is not yet justified to quantify their contribution to

Table 2
Principal reports relevant to singleton freemartinism, classified by the evidence directly reported.

Study	Animals and recorded birth context	Evidence of male-derived cells or DNA	Reproductive phenotype or pathology	Inference supported	Principal limitation
Wijeratne et al. [5]	Five single-born sex-chromosome chimeras among 34 infertile heifers; three examined post mortem	XX/XY leukocyte chimerism	Variable internal abnormalities in all five; ovarian activity in two; primitive ovarian and testicular structures described in one	Strong clinical evidence for pathological singleton freemartinism	Small, selected series; limited gestational documentation; no published gonadal histopathological images
Murakami et al. [6]	One single-birth case within 18 sterile heifers	60,XX/60,XY karyotype	Sterility; case classified by the authors as a single-birth freemartin	Independent cytogenetic evidence in a sterile singleton	Single case; limited phenotypic detail in the report summary
Kadokawa et al. [7]	One molecularly positive animal among 22 singleton females after multiple-embryo transfer	Male-specific DNA detected by PCR; peripheral-blood cytogenetics reported as female	Grossly normal reproductive tract; focal seminiferous-tubule-like structure in the examined left gonad	Singleton freemartinism supported by embryo-transfer context, molecular evidence and focal gonadal differentiation	Single case; contralateral gonad unavailable; no prevalence inference possible
Szczerbal et al. [14]	Twelve single-born heifers selected after culling for internal-tract underdevelopment	Seven had 60,XX/60,XY leukocyte chimerism and detectable <i>SRY</i> and <i>AMELY</i>	Internal reproductive-tract underdevelopment in all selected heifers; no gonadal histopathology	Seven chimeric singletons had a compatible pathological phenotype	Clinically selected sample, clustered source and no unselected population denominator
Garcia-Isperto et al. [15]	Ninety-five single-born females after reduction of twin or triplet pregnancies at 28–34 days	Not tested	Subsequent reproductive outcomes did not differ from 1688 controls	Early reduction of a co-conceptus did not inevitably impair fertility	Sex of reduced conceptus inferred probabilistically; no chimerism or anatomical testing reported
Lindtke et al. [8]	322 F1 cattle; five animals recorded as singletons among 20 putative blood chimeras	Increased heterozygosity in low-pass blood sequencing; discordance absent from hair-bulb SNP data	Not reported for the five singleton animals	Supports blood chimerism and possible lost or unrecorded co-twins among registered singletons	Does not establish reproductive pathology or freemartinism
Maylem et al. [16]	One singleton water buffalo within a cattle and buffalo diagnostic series	<i>SRY</i> and X- and Y-associated amelogenin PCR products	Deformed external genitalia; detailed internal or histopathological assessment was not reported for the singleton	Supports suspected freemartinism in an <i>SRY</i> -positive singleton water buffalo	Supportive cross-species evidence integrating molecular and external morphological findings

unexplained infertility, repeat breeding or reproductive inefficiency in modern cattle populations.

5. Reappraisal of a singleton freemartin born following multiple-embryo transfer

5.1. Original study population and identification of the index animal

Kadokawa et al. [7] investigated 22 singleton female calves born after the transfer of multiple non-sexed embryos. Seventeen were Japanese Black, three were Aberdeen Angus and two were Polled Hereford cattle. Peripheral blood from each animal was examined by conventional chromosome analysis and by PCR targeting *btDYZ*, a repetitive sequence specific to the bovine Y chromosome. One hundred leukocyte metaphases were evaluated per animal. Morphological examination of the genital tract, by vaginal measurement or transrectal palpation, was possible in 19 of the 22 females.

Twenty-one animals had no XY metaphases among the 100 cells examined and were negative for male-specific DNA by PCR. The remaining animal, a 15-month-old Aberdeen Angus female, likewise had no XY metaphases detected but was positive for male-specific DNA in blood. The discordance was interpreted as being compatible with a male-derived blood-cell population below the detection limit of the

metaphase count. In contemporary terminology, however, the PCR finding demonstrates a Y-chromosome sequence in DNA extracted from blood; without cell-level localization, it does not by itself prove XX/XY cellular chimerism. Its diagnostic meaning must therefore be considered together with the gestational record and reproductive morphology.

The developmental history of this animal was unusually well documented. Two excellent-grade frozen-thawed embryos had been transferred non-surgically, one into each uterine horn. Ultrasonography showed the loss of one embryo before day 34 of gestation, and the surviving calf was born as a singleton female. The sex of the lost embryo was not determined. Consequently, the sequence of a heterosexual multiple pregnancy followed by loss of the male conceptus remains an inference, but one supported by the transfer of two non-sexed embryos, ultrasonographically observed embryo loss and subsequent detection of male-specific DNA.

5.2. Clinical and gross reproductive findings

The index animal had externally and internally female genitalia that were reported to be within the normal range in overall form and size. The clitoris was slightly enlarged, measuring approximately 10 mm, but this measurement was considered within the normal range in the original report; the vulvar hair was not elongated. The uterus, cervix and

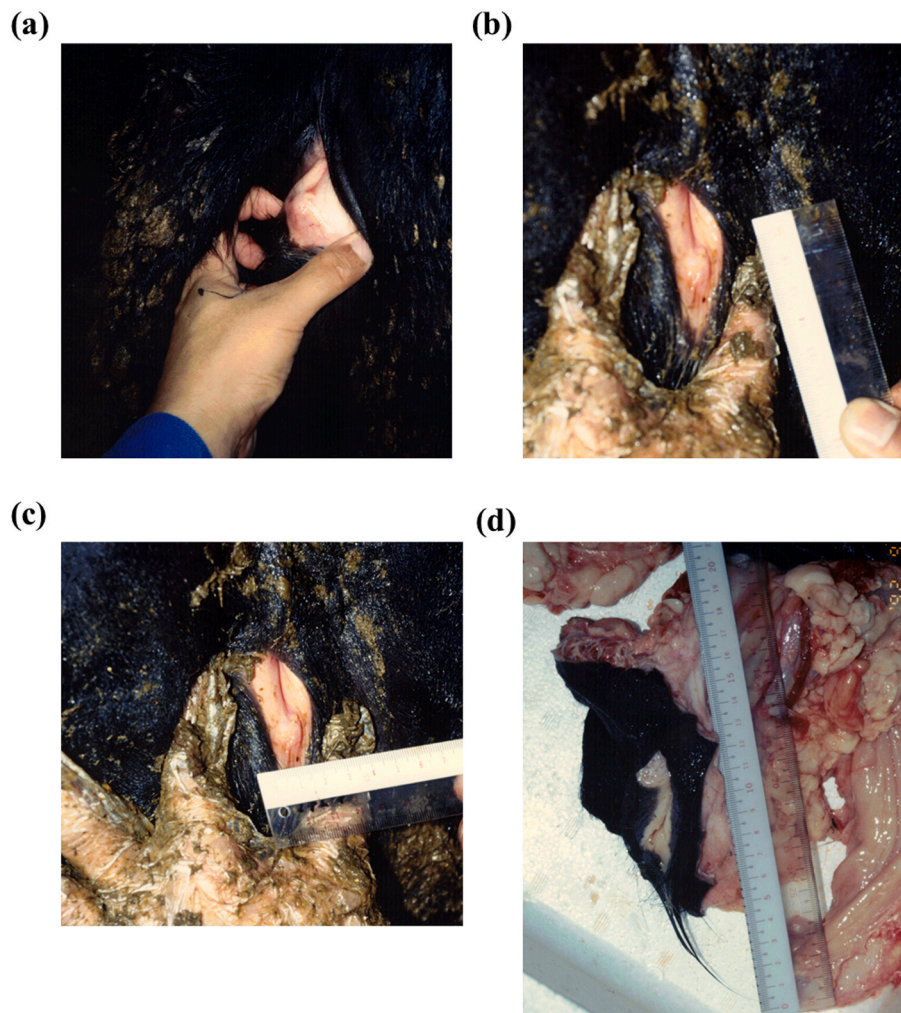


Fig. 1. External genitalia of the singleton female originally reported by Kadokawa et al. [7]. (a) Original color photograph corresponding to Fig. 4 of the original report, which was published in black and white. (b, c) Contemporaneous color photographs obtained during clinical examination, showing the vulva and clitoris with a measuring scale; these images were not included in the original publication. (d) Post-mortem view of the external genitalia. Mild clitoral enlargement was observed, whereas the distribution and length of the perineal hair appeared grossly normal. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

oviducts were present, and neither seminal-vesicle-like structures nor rudimentary Wolffian-duct derivatives were identified. Both uterine horns were approximately 100 mm long and 35 mm in diameter. The animal appeared to exhibit estrous behavior, although fertility was not tested.

The recovered left gonad measured approximately 30×20 mm and was grossly ovarian. Histological examination showed five Graafian follicles and predominantly normal ovarian tissue. Within that ovary, however, there was a small focus, with a maximum diameter of approximately 1.5 mm, composed of structures resembling seminiferous tubules [7]. This combination is important: the grossly near-normal phenotype and preserved follicular structures show that marked external masculinization or severe Müllerian-tract hypoplasia is not required for focal testicular differentiation to occur.

Fig. 1 presents the external genitalia using the color version of the image published in 1995 and additional contemporaneous color photographs. Fig. 2 presents the gross reproductive tract and recovered gonad. These images document the comparatively mild external phenotype and the largely female gross anatomy. They should not be interpreted as demonstrating a wholly normal reproductive system, because histological examination identified focal gonadal abnormality and reproductive function was not established.

5.3. Reappraisal of the archival histopathological material

The original article reproduced only a limited number of black-and-white photomicrographs. The retained archive contains the original color photographs and additional histological fields from the same investigation; a selected set of these materials has also been deposited in the Yamaguchi University Institutional Repository for preservation and verification [17]. No new animal procedures were undertaken for the present reappraisal.

Fig. 3 places the low-magnification cross-section of the left gonad alongside higher-magnification and additional sections. Antral follicles with recognizable follicular architecture establish the ovarian component. The archived sections contain organized profiles whose cellular arrangement on hematoxylin-eosin staining and tubule-like configuration on periodic acid-Schiff staining are consistent with seminiferous tubule-like differentiation. Fig. 4 shows comparable structures in additional sections or fields. These images document the morphology in more than one archived view, but the available material does not determine whether every image represents a distinct focus or a different section or field of the same focus.

The coexistence of ovarian follicles and seminiferous tubule-like structures within the same gonad supports its classification as an ovotestis on histomorphological grounds. The material adds direct visual documentation to the molecular and gestational evidence and is the principal added value of revisiting the 1995 case. Within the bovine

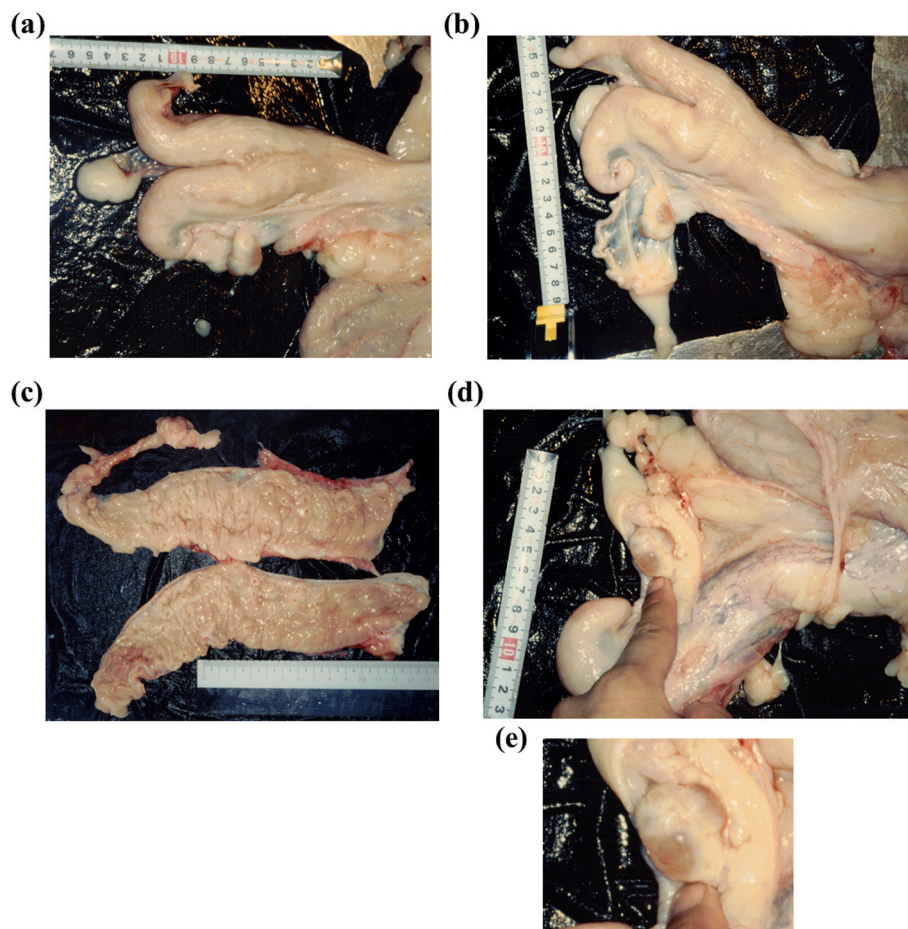


Fig. 2. Gross morphology of the reproductive tract and recovered left gonad of the singleton female originally reported by Kadokawa et al. [7]. (a) Original color photograph corresponding to Fig. 3a of the original report, which was published in black and white. (b) Additional photograph showing the uterine horn and oviduct. (c) Original color photograph corresponding to Fig. 3b of the original report, which was published in black and white. (d) Additional photograph showing the recovered left gonad and associated reproductive structures. (e) Higher-magnification view of the gonad shown in (d). The right gonad was lost at the slaughterhouse and was unavailable for examination. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

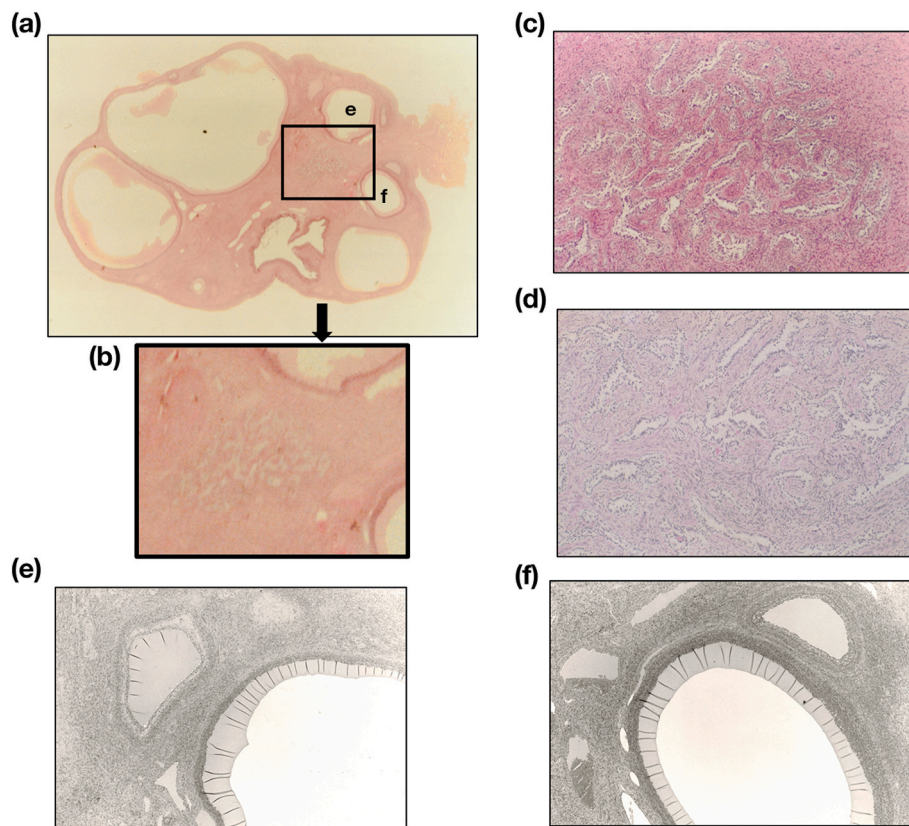


Fig. 3. Histological features of the recovered left gonad. (a) Original low-magnification photomicrograph from Kadokawa et al. [7], showing the ovary and the area containing a seminiferous tubule-like focus (rectangle). (b) Higher-magnification view of the tubule-like focus in (a), hematoxylin-eosin staining. (c) Additional hematoxylin-eosin-stained section showing seminiferous tubule-like structures. (d) Additional periodic acid-Schiff-stained section showing comparable tubule-like structures. (e, f) Antral follicles visible in the ovarian tissue, with a granulosa cell layer and antral cavity. The coexistence of ovarian follicles and seminiferous tubule-like structures supports focal ovotesticular differentiation. Panels (c-f) are archival images not included in the original publication.

singleton literature identified by the search described in Section 2, we found no earlier report providing a comparably documented set of histopathological images showing both ovarian and testis-like differentiation in the same gonad.

This interpretation should nevertheless remain within the resolution of the archived material. The original study did not use immunohistochemical markers of Sertoli-cell or Leydig-cell differentiation, such as SOX9, AMH or steroidogenic markers, and the proportion of the gonad occupied by the tubule-like component was not quantified. Multiple photomicrographs strengthen the morphological observation but do not establish that testicular differentiation was widespread throughout the gonad. The appropriate conclusion is focal ovotesticular differentiation, not complete gonadal sex reversal.

5.4. Integrated interpretation

Considered separately, none of the three principal observations reconstructs the entire developmental sequence. The transfer record and early embryo loss establish that two conceptuses were initially present but not that the lost embryo was male. The blood PCR result establishes the presence of a Y-chromosome-specific sequence but does not localize XY cells or quantify their abundance. The gonadal histology establishes focal testis-like differentiation within an ovary but does not identify the chromosomal constitution of the cells forming that focus. Considered together, however, these independent observations support a coherent and biologically plausible interpretation: a male co-conceptus was present long enough for fetal exchange and developmental influence to occur and was then lost, leaving a singleton female with male-specific DNA in blood and focal ovotesticular differentiation.

This synthesis supports the original classification while illustrating why a diagnosis should not rest on blood chimerism alone. The strongest evidence of pathological freemartinism in this animal is the reproductive phenotype at the tissue level, interpreted in the context of the documented multiple-embryo pregnancy and molecular finding. The case also demonstrates phenotypic heterogeneity. A female may have a uterus, oviducts, cervix, near-normal external genitalia and ovarian follicles while still containing a small focus of testicular differentiation. It does not follow that other singleton females positive for the same Y-chromosome-specific sequence, or other blood-chimeric singleton cattle, have the same gonadal lesion.

The case cannot establish the prevalence of singleton freemartinism, its contribution to herd infertility or whether current reproductive technologies have increased its occurrence. Indeed, 21 of the 22 singleton females in the original multiple-embryo-transfer series were negative by both assays, and the authors concluded that most such females would be expected to have normal reproductive function [7]. The appropriate contemporary significance of the index case is therefore evidential rather than epidemiological: it demonstrates a biologically coherent pathway and provides tissue-level documentation of one possible outcome.

5.5. Limitations of the historical case

Several limitations restrict interpretation and should be explicit.

1. **Single affected animal.** The index animal cannot provide an estimate of prevalence, risk or economic importance.

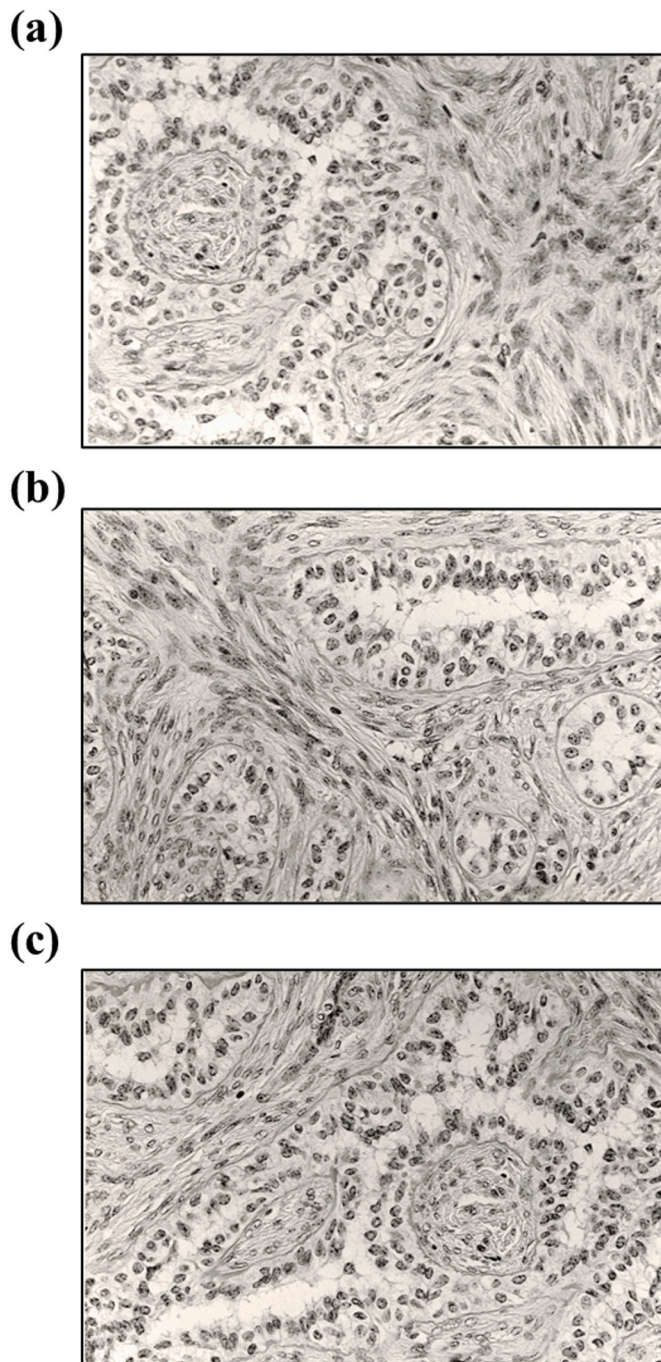


Fig. 4. Additional histological fields containing seminiferous tubule-like structures in the recovered left gonad. (a) Original photomicrograph from Kadokawa et al. [7], hematoxylin-eosin staining. (b, c) Additional hematoxylin-eosin-stained sections or fields from the same gonad. Panels (b, c) are archival images not included in the original publication. These images document the tubule-like morphology in more than one field but do not quantify its distribution within the gonad.

2. Incomplete gonadal examination. The right gonad was lost at the slaughterhouse and was unavailable for gross or histological examination. Whether it was a normal ovary, an ovotestis or a more extensively masculinized gonad is unknown; the findings from the left gonad cannot be assumed to represent both sides, and any relationship between the cellular composition of the right gonad and the molecular finding in blood cannot be evaluated.

- 3. Incomplete reconstruction of the vanished conceptus.** Although two embryos were transferred and one was lost before day 34, its sex was not determined and placental vascular communication was not demonstrated directly.
- 4. Blood result without cellular localization.** Male-specific DNA was detected by PCR, but no XY metaphase was found among 100 leukocytes. The original study did not use interphase fluorescence in situ hybridization (FISH) to localize rare XY cells or quantitative PCR or droplet digital PCR to estimate the abundance of Y-chromosome material.
- 5. Histomorphological rather than immunophenotypic classification.** The testis-like component was identified from conventional histology and PAS staining; lineage-specific immunohistochemistry or molecular characterization was not performed.
- 6. Unknown fertility.** Apparent estrous behavior, a grossly female tract and follicular development do not establish reproductive competence. The animal was not subjected to a breeding trial.
- 7. Archival sampling.** The retained images document additional sections and fields but do not constitute systematic serial-section mapping of the entire gonad. They cannot determine the total volume or spatial distribution of the testis-like component.

Within these limitations, the concordant gestational, molecular and morphological observations establish focal ovotesticular differentiation in one male-specific-DNA-positive singleton female after documented loss of one of two transferred embryos. They do not establish the sex of the lost embryo, cellular XX/XY chimerism in blood or gonad, bilateral gonadal morphology, fertility, or population frequency. These unresolved questions guide the prospective diagnostic investigations proposed later in this review.

6. Twinning, conceptus loss and the unresolved prevalence of singleton freemartinism

6.1. Twinning creates an opportunity, not an estimate of disease frequency

Freemartinism requires a biological context in which a female conceptus can be exposed to cells and developmental signals from a male co-conceptus through a shared placental circulation. Factors that increase dizygotic twinning therefore increase the number of pregnancies in which classical freemartinism could arise. They may also increase the number of pregnancies in which a female is ultimately born alone after loss of a co-conceptus. Neither proposition establishes how often a surviving singleton develops reproductive pathology.

Most twin pregnancies in dairy cattle are dizygotic and arise following multiple ovulation. In lactating dairy cows, the incidence of multiple ovulation was strongly associated with milk production in a prospective observational study [18]. At the population level, twinning in Holstein cattle has increased over time concurrently with increases in milk production, although parity, genetics, season, management and reproductive protocols also affect risk [19]. These observations justify considering high-producing dairy populations when designing surveillance. They do not demonstrate that milk yield itself causes singleton freemartinism, nor do they show that the prevalence of the condition has increased.

The distinction can be expressed as a sequence of conditional events. A pregnancy must contain at least one male and one female conceptus; a functional vascular connection must develop early enough for cellular exchange and developmental disruption; the male conceptus must subsequently be lost while the female survives; and the surviving female must develop a reproductive abnormality rather than blood chimerism alone. A change in the frequency of the first event cannot be translated directly into a change in the final outcome without probabilities for the intervening events. Those probabilities have not been measured prospectively for singleton freemartinism.

6.2. Selective conceptus loss is biologically possible but poorly quantified for this outcome

Loss of one member of a bovine twin pregnancy is well recognized, whether spontaneous or induced. In a slaughterhouse study of 65 twin pregnancies, Agerholm et al. [20] identified four pregnancies with pre-slaughter fetal mortality. Both fetuses were dead in three pregnancies, whereas only one of two approximately 40-day-old fetuses showed evidence of death in the fourth. This observation demonstrates that selective death of one twin can occur. Because the material was collected cross-sectionally at slaughter, it does not show that the surviving conceptus would have progressed to term, that the dead conceptus would have disappeared completely, or that a surviving female would have developed freemartinism.

Clinical studies of intentional twin reduction provide additional evidence that a twin pregnancy can result in a continuing singleton pregnancy. Andreu-Vázquez et al. [21] assigned 55 cows with two live embryos at days 35–41 either to manual amnion rupture with progesterone support or to an untreated control group. Pregnancy loss before day 90 was common in both groups and depended strongly on whether the twins were unilateral or bilateral. The study was designed to assess pregnancy maintenance, not freemartinism, but it illustrates that laterality, gestational timing and the method of reduction influence whether a singleton survives after a twin is lost.

The most directly relevant follow-up is the study by Garcia-Ispuerto et al. [15]. Ninety-five single-born females that remained after manual reduction of twin or triplet pregnancies at days 28–34 survived to 12 months of age and entered the artificial-insemination period. Their pregnancy status by 15 months of age, culling for infertility and calving before 24 months did not differ from those of 1688 contemporaneous control heifers. The sex of the reduced conceptus was not determined, and cytogenetic or molecular chimerism was not reported; exposure to a male co-conceptus was therefore inferred for a proportion of the group rather than documented individually. In addition, because inclusion was conditional on survival to 12 months and entry into the breeding program, the study did not evaluate reproductive development in female offspring that died or were excluded before that stage. Even with those limitations, the absence of a detectable reproductive disadvantage among the females examined is important. Early presence and subsequent removal of a co-conceptus do not inevitably produce a freemartin phenotype.

These studies collectively support the feasibility of a vanished-co-twin pathway but do not provide its probability. For pathological singleton freemartinism to occur, loss must take place after biologically consequential communication has developed but early enough for the male conceptus or its remnants not to be recognized at parturition. The duration of that interval and the probability of each step remain unknown.

6.3. Embryo transfer and other reproductive interventions

Transfer of more than one non-sexed embryo can deliberately create a multiple-conceptus pregnancy and therefore an opportunity for heterosexual vascular communication. The 1995 multiple-embryo-transfer series is informative because the full denominator was reported: among 22 singleton females, 21 were negative by chromosome analysis and male-specific PCR, whereas one was PCR-positive and had focal ootesticular differentiation [7]. Thus, the study demonstrates that the proposed sequence can occur after multiple-embryo transfer, but it also shows that the great majority of singleton females in that small series had no evidence of male-derived DNA by the methods used.

The frequency observed in this historical series should not be applied to contemporary embryo-transfer programmes. The sample was small, embryo-transfer practices and diagnostic technologies have changed, and a PCR-positive animal was not equivalent to a prospectively defined clinical endpoint. Furthermore, transfer of multiple embryos does not

establish that all embryos implanted, that they were of opposite sex, that vascular anastomosis developed, or that one was subsequently lost after the critical developmental interval.

Reproductive technologies may therefore alter the opportunity structure through the number, location and sex of conceptuses and through the timing of reduction or loss. Evidence that they have increased the population prevalence of singleton freemartinism is not currently available. The appropriate clinical inference is awareness of a plausible developmental history, not a presumption that a singleton female produced by embryo transfer is affected.

6.4. Why existing singleton studies cannot establish prevalence

The principal published datasets answer different questions and do not share a population denominator. Wijeratne et al. [5] began with heifers selected for failure to conceive. Szczerbal et al. [14] began with 12 single-born heifers already culled for underdevelopment of the internal reproductive tract. Kadokawa et al. [7] examined a small, intervention-defined group of singleton females after multiple-embryo transfer. Lindtke et al. [8] screened blood sequencing data from 322 F1 cattle and identified five animals recorded as singletons with genomic evidence compatible with blood chimerism, but reproductive anatomy and fertility were not reported for those animals. These designs cannot be combined to calculate a prevalence of pathological singleton freemartinism.

In particular, the seven chimeric animals among the 12 heifers studied by Szczerbal et al. [14] represent the proportion within a selected series of single-born heifers with known reproductive-tract underdevelopment, not the proportion among all singleton females. Similarly, the five putative blood chimeras recorded as singletons by Lindtke et al. [8] cannot be counted as five singleton freemartins because the study did not demonstrate reproductive pathology. The numerator and denominator required for a prevalence estimate are absent in both situations.

The number of published case reports is also uninformative about population frequency. It depends on whether a birth was observed and recorded accurately, whether reproductive abnormalities became clinically apparent, whether specialized testing was available, whether an animal underwent post-mortem examination and whether the findings were published. Under-ascertainment is therefore plausible, but its magnitude is unknown. The current literature supports a hypothesis of incomplete recognition, not a conclusion that a large hidden population exists.

6.5. Implications for reproductive performance and herd management

Singleton freemartinism may reasonably be included in the differential diagnosis of a single-born heifer with congenital reproductive-tract abnormalities, masculinized genitalia, unexplained absence of puberty or sterility, particularly when early ultrasonography or embryo-transfer records indicate a previous multiple pregnancy. This is a targeted diagnostic indication. It does not justify attributing repeat breeding, low fertility or unexplained infertility generally to freemartinism. Those outcomes have numerous more common infectious, anatomical, endocrine, genetic and management-related causes.

The available evidence also does not quantify an economic burden attributable specifically to singleton freemartinism. Twin pregnancies themselves have well-documented adverse clinical and economic consequences in dairy cattle, including greater risks of pregnancy loss, dystocia, perinatal mortality and subsequent reproductive problems [19]. Those established costs should not be reassigned to an unquantified singleton outcome. Any herd-level claim would require confirmation of both the developmental disorder and its causal relation to reproductive failure in individual animals.

Accordingly, modern production trends should be presented as reasons to investigate rather than as evidence of increased disease

prevalence. Greater use of early ultrasonography, retention of embryo-transfer and pregnancy-reduction records, and application of a staged diagnostic pathway could make prospective assessment feasible. The diagnostic methods and their appropriate interpretation are considered in Section 7.

6.6. Requirements for a valid prevalence study

A defensible estimate would require prospective enrollment of an unselected or explicitly defined cohort of pregnancies, followed through birth and the subsequent reproductive development of all singleton female offspring. Pregnancy should be examined early and repeatedly by ultrasonography to document conceptus number, laterality, viability, time of loss and, where feasible, conceptus sex. Embryo-transfer details should include the number and sex status of embryos transferred. After birth, testing should distinguish Y-chromosome sequence detection from cell-level XX/XY chimerism and should be paired with standardized examination of the external and internal reproductive tract, gonadal structure, endocrine function and subsequent fertility.

Cases should be classified in advance using the levels of evidence defined in Section 3: evidence of prior multiple gestation, fetal cell exchange or blood chimerism, reproductive developmental abnormality and integrated diagnostic status. Reporting molecularly positive animals without phenotype data, or phenotypically abnormal animals without an appropriate differential diagnosis, would reproduce the present uncertainty. Multicenter enrollment would be needed because twinning, reproductive management and recording practices differ among herds.

Until such data are available, the true prevalence of singleton freemartinism and its quantitative contribution to reproductive failure remain unknown. Current historical, clinical and genomic observations justify prospective investigation, but not the conclusion that singleton freemartinism is an established or increasing cause of reproductive inefficiency in modern cattle populations.

7. Diagnostic evaluation of suspected singleton freemartinism

7.1. Diagnostic objective

Freemartin diagnosis has long combined reproductive history with clinical and laboratory findings [3]. The purpose of investigating a suspected singleton freemartin is not merely to detect a Y-chromosome sequence in blood. The diagnostic process must address two distinct questions: whether male-derived cells or DNA are present, and whether the female reproductive system has undergone abnormal development compatible with freemartinism. Neither question can substitute for the other. Depending on the method, a blood-based result documents either Y-bearing cells or a Y-chromosome sequence in the sampled blood, whereas clinical examination, imaging and histopathology assess the reproductive phenotype. The most defensible diagnosis therefore integrates the available gestational history, the precisely defined cytogenetic or molecular finding and evidence of reproductive developmental abnormality.

Investigation is most appropriate when a single-born female has congenital genital abnormalities, failure of normal pubertal development or sterility, or when early pregnancy or embryo-transfer records indicate that another conceptus was previously present. Routine testing of all females with repeat breeding or low fertility is not supported by current evidence. In animals with an unequivocally recorded male co-twin, early blood testing can inform further reproductive assessment instead of assuming that every female is infertile solely from the birth history.

7.2. Clinical history and examination

The first step is to reconstruct the developmental context. Records should include the number and location of conceptuses on early

ultrasonography, subsequent conceptus loss, the number and sex status of embryos transferred, placental or fetal remnants at calving and the reliability of the recorded singleton birth. Absence of a male calf at parturition is not evidence that no male conceptus was present earlier in gestation.

External examination should document vulval development, clitoral size, anogenital distance and any male-like structures. Vaginal examination or vaginoscopy can identify reduced vaginal length, blind termination or an absent or non-patent cervix. However, normal external genitalia, estrous behavior or even ovarian activity do not exclude freemartin-associated internal abnormalities. Wijeratne et al. [5], for example, described singleton chimeric heifers with apparently normal external or clinical findings but non-patent cervical or uterine structures at post-mortem examination.

Transrectal palpation and ultrasonography can assess the cervix, uterine horns and gonadal location and morphology. These methods are clinically accessible in animals of sufficient size and can identify uterine hypoplasia, cord-like horns, absent or atypical gonads and structures resembling testes or ovotestes. Their limitations are operator dependence, age and body-size constraints, and the difficulty of characterizing small or ectopic gonads. Imaging demonstrates anatomy, not its developmental cause; similar findings can occur in other differences of sex development, including loss of one X chromosome (X monosomy), abnormal gonadal development in an XY animal (XY gonadal dysgenesis) and testicular or ovotesticular disorders without detectable SRY [22].

7.3. Cytogenetic and molecular detection of male-derived cells or DNA

7.3.1. Karyotyping and fluorescence in situ hybridization

Conventional karyotyping of cultured peripheral-blood lymphocytes directly identifies individual 60,XX and 60,XY metaphases and has historically provided the reference evidence for leukocyte chimerism. It can also reveal alternative chromosomal diagnoses. Its disadvantages are the need for viable cells, cell culture, skilled chromosome preparation and analysis of a sufficient number of metaphases. A negative result means that no XY metaphase was observed among the cells examined; it does not exclude an XY population below the sampling limit. This limitation is illustrated by the occasional phenotypically affected animal in which repeated examination of hundreds of metaphases detected only 60,XX cells [23].

Fluorescence in situ hybridization (FISH), which uses a labeled DNA probe to locate a specific chromosome sequence within individual cells, can examine interphase nuclei as well as metaphase chromosomes. Sohn et al. [24] found close agreement between Y-chromosome FISH and karyotyping in young heterosexual twins. FISH retains the principal advantage of cell-level localization, but requires validated probes, fluorescence microscopy and specialist interpretation. Both karyotyping and FISH usually assess blood leukocytes and therefore establish leukocyte chimerism, not the chromosomal composition or differentiation of the reproductive tract.

7.3.2. Qualitative PCR and loop-mediated isothermal amplification

PCR assays targeting Y-linked sequences such as SRY (sex-determining region Y), AMELY (Y-linked amelogenin) or other male-specific loci provide rapid and sensitive detection using DNA extracted from blood. Inclusion of an independent amplification control and appropriate male, female and no-template controls is essential. A positive result establishes that the target sequence is present in the specimen. Because extracted DNA is analyzed rather than individual cells, qualitative PCR alone does not demonstrate an XX/XY cellular mixture, quantify its proportion or determine whether male-type reproductive development occurred. Very low-level positives should be confirmed from a newly collected specimen and, preferably, with a second Y-linked target or a cell-based method to reduce the risk of contamination or locus-specific error.

Loop-mediated isothermal amplification (LAMP) provides a simpler, rapid alternative when conventional thermal cycling is unavailable. Hirayama et al. [25] detected male-specific DNA in diluted peripheral blood and reported detection of an XY-leukocyte contribution as low as 0.01% under their experimental conditions. Its speed and minimal equipment requirements favor field-oriented screening. Nevertheless, high analytical sensitivity increases the importance of contamination control, and a qualitative LAMP-positive result has the same biological limitation as qualitative PCR: it indicates a male-specific sequence in the tested blood sample but does not establish pathological freemartinism.

7.3.3. Quantitative PCR, droplet digital PCR and genome-wide approaches

Quantitative PCR (qPCR) and droplet digital PCR (ddPCR) can estimate the relative abundance of Y-linked DNA. Qiu et al. [26] compared qualitative SRY PCR, relative SRY quantification and measurement of the male-associated H-Y antigen in 12 heterosexual twin females and showed the feasibility of relating a quantitative molecular signal to subsequent fertility observations. Maylem et al. [16] subsequently applied qualitative PCR and qPCR targeting SRY in cattle and water buffalo, including an SRY-positive singleton water buffalo with deformed external genitalia. These studies extend the practical evidence for PCR-based investigation and demonstrate the value of recording phenotype and reproductive outcome alongside the molecular result.

Numerical thresholds reported for relative SRY content should be interpreted within the particular assay, calibrator, target, specimen and study population in which they were derived. They are not yet universal cut-offs for fertility or for the severity of masculinization. The proportion of male-derived DNA in blood is also not a measure of the timing or concentration of developmental signals received by the reproductive tract. Accordingly, qPCR can support and quantify detection of male-derived DNA, but should not be used alone to predict reproductive anatomy or fertility.

Using copy numbers for X-linked amelogenin (*AMELX*) and Y-linked amelogenin (*AMELY*), Szczerbal et al. [27] showed that ddPCR detected and quantified leukocyte chimerism in 22 cytogenetically confirmed Holstein Friesian freemartins, including samples with a low-frequency male cell line. Partitioning of the reaction and absolute copy-number estimation can improve precision and reduce dependence on an external calibration curve. However, instrumentation and assay validation limit routine availability, and the resulting X:Y DNA ratio remains a measurement in the sampled tissue rather than a direct test of gonadal pathology.

Short tandem repeats (STRs), single-nucleotide polymorphism (SNP) arrays and sequencing can detect the presence of two genetic contributors and may clarify whether an apparent excess of genetic differences represents blood chimerism. Kozubska-Sobocińska et al. [28] combined karyotyping, Y-linked PCR, STR analysis and relative qPCR in 24 Holstein Friesian heifers born with a male co-twin, illustrating the complementary information obtained from cytogenetic and molecular tests. Comparison of blood with a non-hematopoietic specimen is particularly informative: Lindtke et al. [8] detected putative chimerism in blood sequencing data that was absent from hair-bulb SNP-array data. Such discordance supports restriction of the second cell population to blood. Conversely, detection of Y-linked material in a non-blood tissue requires careful evaluation for an XY chromosome constitution present throughout the animal, a mixture of chromosome constitutions within the animal (mosaicism), tissue chimerism and contamination. Genome-wide approaches can be valuable in unresolved cases, but are less suitable than targeted assays for routine first-line testing.

7.4. Endocrine assessment

Anti-Müllerian hormone (AMH) is biologically relevant because exposure to AMH from the male co-conceptus contributes to Müllerian duct regression. Its diagnostic interpretation after birth is strongly age dependent. Rota et al. [29] reported very high plasma AMH

concentrations in newborn males and freemartins, whereas concentrations in normal females were substantially lower. In freemartins, however, AMH declined sharply during the first fortnight and reached the female range, supporting a predominantly male-co-twin origin of the neonatal circulating hormone.

AMH may therefore provide useful adjunctive evidence in the immediate neonatal period or indicate functioning Sertoli-like tissue in selected animals, but a low concentration in an older heifer cannot exclude freemartinism. Conversely, an increased concentration is not specific for the vanished-male pathway and must be interpreted with age, assay-specific reference data and gonadal imaging. Testosterone, progesterone and estradiol measurements may help characterize gonadal function or alternative disorders, but none independently demonstrates prior fetal exchange. Endocrine testing is consequently complementary rather than confirmatory.

7.5. Histopathology and tissue localization

Histopathological examination is the most direct means of defining an abnormal gonadal phenotype. It can distinguish ovarian follicles, seminiferous-cord- or tubule-like structures, Sertoli-like cells, Leydig-like interstitial cells and mixed ovotesticular differentiation. Immunohistochemistry for markers such as SOX9, AMH and steroidogenic proteins can strengthen cellular identification when suitable fixed tissue is available [12,30]. Histopathology does not by itself establish that a vanished male conceptus caused the lesion, and a unilateral specimen cannot define the contralateral gonad. It is also invasive and is generally restricted to biopsy, gonadectomy or post-mortem material, so it cannot serve as a routine screening test.

Where the mechanism remains uncertain, localization of X- and Y-bearing cells by FISH or other validated methods in gonadal or reproductive-tract tissue can supplement morphology. Tissue-level XY cells must still be distinguished from constitutional or mosaic sex-chromosome abnormalities. The result should therefore be integrated with blood findings, gestational history and the distribution of chromosomal or molecular signals across tissues.

7.6. A staged diagnostic pathway

A practical investigation can proceed in five stages.

1. **Establish the indication and history.** Confirm the recorded birth status and retrieve early ultrasonographic, embryo-transfer, pregnancy-reduction and calving records. Document the clinical reason for testing.
2. **Define the reproductive phenotype.** Examine external genitalia and vaginal patency and, when age and size permit, assess the cervix, uterus and gonads by palpation and ultrasonography. Record puberty and fertility outcomes when available.
3. **Test blood for male-derived cells or DNA.** Use a validated Y-linked PCR assay for accessible first-line screening. Use karyotyping or interphase FISH when cell-level confirmation is required, and a validated quantitative assay such as ddPCR when the abundance of sex-chromosome targets is relevant. Amplification-based assays require appropriate positive, negative and internal amplification controls.
4. **Resolve discordant or atypical findings.** Repeat low-level molecular positives on a new specimen, use a second target or method, and compare blood with an appropriate non-hematopoietic tissue when the distinction between blood-restricted chimerism and another sex-chromosome constitution is important. Investigate alternative differences of sex development when phenotype and chimerism results are discordant.
5. **Assign an evidence-based classification.** Report separately the gestational evidence, the molecular or cytogenetic finding, the reproductive phenotype and the integrated diagnosis. A singleton

female with only a positive blood test should be described as having male-derived DNA or XX/XY blood chimerism, according to the method used, rather than as a definitive singleton freemartin. Pathological singleton freemartinism requires compatible reproductive developmental abnormality supported by the available gestational and chimerism evidence.

This staged approach is intended to prevent both overdiagnosis from an isolated molecular result and under-description of animals with genuine reproductive pathology. Its value in herd practice lies principally in selecting the right animals for testing and avoiding repeated breeding attempts only after an irreversible reproductive abnormality has been demonstrated. The present evidence does not support untargeted population-wide testing or estimation of herd-level economic benefit.

7.7. Comparative interpretation of diagnostic methods

The specimens, timing, evidential meaning, advantages and principal limitations of the diagnostic approaches discussed above are compared in Table 3.

8. Conclusions and research priorities

8.1. Conclusions

The available evidence supports the existence of singleton freemartinism in cattle, but not an estimate of its prevalence. Historical reports describe females born without a male co-twin at parturition in which XX/XY leukocyte chimerism occurred together with congenital abnormalities of the reproductive tract or gonad. These cases establish biological possibility. They do not show how frequently the condition occurs among singleton females or how much it contributes to reproductive failure in cattle populations.

The historical case reported by Kadokawa et al. [7] provides one

well-documented example following multiple-embryo transfer. The original observations combined a recorded early loss of one of two transferred embryos, detection of male-specific DNA in blood and focal testis-like differentiation within the recovered left ovary. The additional archival histological images presented here strengthen the morphological documentation of focal ovotesticular differentiation. Interpretation nevertheless remains limited by the unknown sex of the lost embryo, the absence of cell-level localization of the male-specific signal, loss of the right gonad before examination, lack of immunophenotyping and unknown fertility. The case should therefore be regarded as an important component of the wider evidence base, not as a basis for epidemiological inference.

The central diagnostic distinction is between evidence of fetal cell exchange and evidence of pathological reproductive development. In an appropriate developmental context, persistent XX/XY blood chimerism is evidence that fetal cell exchange occurred and directly demonstrates two sex-chromosome cell populations in blood, whereas detection of a Y-linked sequence by PCR demonstrates male-specific DNA in the extracted specimen. When analytically validated, either finding can strongly support a diagnosis in an appropriate biological and clinical context. Neither blood chimerism nor detection of a Y-linked sequence establishes that masculinization, Müllerian-tract disruption or gonadal sex reversal occurred. Conversely, an abnormal reproductive phenotype requires differential diagnosis because other chromosomal and developmental disorders can produce similar findings. A diagnosis of singleton freemartinism is consequently strongest when gestational evidence, a precisely characterized cytogenetic or molecular finding and compatible reproductive pathology converge.

Factors encountered in modern dairy production, including double ovulation, multiple pregnancy and transfer of more than one embryo, may increase the number of gestations in which the necessary developmental sequence could occur. This represents an increased opportunity, not demonstrated increased prevalence. Available studies of planned embryo reduction show that the previous presence and subsequent loss of another conceptus do not inevitably impair the

Table 3
Comparative interpretation of diagnostic methods for suspected singleton freemartinism.

Method	Principal specimen and timing	What a positive result establishes	Main advantages	Principal limitations and appropriate role
Birth and pregnancy records	Dam records; pregnancy to calving	Prior multiple gestation or recorded co-twin	Non-invasive; reconstructs biological opportunity	Early loss and sex may be missed; establishes context, not chimerism or pathology
External examination and vaginoscopy	Live calf or heifer; age affects access	Observable genital or vaginal abnormality	Rapid and inexpensive	Normal findings do not exclude internal lesions; phenotype only
Palpation and ultrasonography	Live heifer, when body size permits	Abnormal cervix, uterus or gonadal morphology	Defines clinically relevant anatomy	Operator- and age-dependent; abnormalities are not specific for freemartinism
Karyotyping	Viable blood lymphocytes; any postnatal age	60,XX and 60,XY metaphases in the cells examined	Direct cell-level evidence; can reveal other chromosome abnormalities	Culture and expertise required; sensitivity depends on metaphase number; describes the sampled blood-cell population only
Interphase FISH	Blood cells or tissue nuclei	Y-positive cells and their frequency in the specimen	Cell localization; no metaphase requirement	Specialist probe and microscopy; tissue sampled determines interpretation
Qualitative Y-linked PCR	Usually whole-blood DNA; any postnatal age	Presence of the target Y sequence in extracted DNA	Rapid, sensitive and widely accessible	Does not localize cells or quantify chimerism; contamination control and confirmation required
LAMP	Whole blood or extracted DNA	Presence of the target male-specific sequence	Rapid; limited equipment; potential field screening	Usually qualitative; very high sensitivity increases contamination risk; does not establish pathology
qPCR	Blood DNA or, in some protocols, cDNA	Relative abundance of a Y-linked target	Quantitative and accessible in molecular laboratories	Assay-dependent calibration; no universal fertility or severity threshold
ddPCR	Blood DNA	Absolute or relative X- and Y-target copy numbers	Sensitive quantification without a standard curve	Specialized equipment; DNA ratio is not a measure of reproductive masculinization
STR, SNP-array or sequencing analysis	Blood, ideally compared with non-blood tissue	Evidence of two genetic contributors or blood-restricted genotype mixture	Genome-wide evidence; can resolve selected discordant cases	Cost, analysis and reference requirements; not routine first-line testing
AMH and other hormones	Serum or plasma; interpretation is age dependent	Endocrine activity compatible with male or Sertoli-like tissue	Useful adjunct in selected neonatal or gonadal evaluations	AMH falls rapidly after birth in freemartins; values are not specific for the mechanism
Histopathology with optional immunohistochemistry	Gonadal or tract tissue after biopsy, surgery or death	Nature and distribution of reproductive-tissue abnormality	Strongest direct characterization of the pathological phenotype	Invasive; sampling may be unilateral or focal; does not reconstruct gestational history

reproductive performance of the surviving female [15]. Likewise, genomic evidence of blood chimerism in cattle recorded as singletons cannot be converted into a count of singleton freemartins without reproductive-phenotype data [8]. The present literature therefore does not establish singleton freemartinism as an overlooked or increasing quantitative cause of reproductive inefficiency.

8.2. Research priorities

The principal need is a prospective, multicenter study beginning during early pregnancy rather than after infertility has been recognized. Repeated ultrasonography should document conceptus number, laterality, viability and time of loss. Where possible, conceptus sex and embryo-transfer details should be recorded. All singleton female offspring should then undergo predefined examination of reproductive anatomy, blood-based testing and follow-up through puberty and breeding. This design would provide the numerator and denominator required to estimate prevalence and would avoid the selection bias inherent in series assembled from infertile or anatomically abnormal heifers.

Diagnostic studies should compare methods against explicitly defined outcomes. Karyotyping and interphase FISH identify cell-level XX/XY chimerism; qualitative PCR and LAMP identify Y-linked sequences; qPCR and ddPCR estimate the abundance of selected targets; and imaging, endocrine assessment and histopathology characterize different components of the reproductive phenotype. Future studies should report analytical sensitivity, controls, specimen type, animal age and concordance among methods. Quantitative molecular values should not be treated as universal predictors of anatomy or fertility until thresholds have been validated independently across laboratories and populations. The integration of molecular findings with phenotypic and reproductive records in recent work, including Maylem et al. [16], provides a useful basis for such validation.

Finally, future case reports should use standardized terminology and report negative as well as positive findings. A female recorded as a singleton with only a Y-linked blood signal should be classified as a molecularly positive or blood-chimeric singleton female, according to the method used. The term *singleton freemartin* should be reserved for animals with compatible reproductive developmental abnormality supported by the available gestational and cytogenetic or molecular evidence. Applying this distinction prospectively would help determine whether singleton freemartinism is merely documented infrequently or is genuinely under-recognized, while avoiding conclusions that extend beyond the current evidence.

CRedit authorship contribution statement

Hiroya Kadokawa: Conceptualization, Investigation, Methodology, Resources, Data curation, Visualization, Writing – original draft, Writing – review and editing.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the author used ChatGPT (OpenAI) to assist with literature searching and English-language editing. The author reviewed and edited the output and takes full responsibility for the content of the article.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

I am grateful to my coauthors of the 1995 study, particularly Dr. Takayoshi Kariya, who taught me blood sampling and transrectal examination in cattle, and Dr. Mitsuru Minezawa, who taught me PCR techniques early in my career. The singleton female case reappraised in this review arose from an embryo transfer performed by Dr. Yusukey Yamamoto, then of the Animal Biotechnology Section, Hokkaido Prefectural Shintoku Animal Husbandry Experiment Station. I thank them again for their contributions to the original study.

References

- [1] Lillie FR. The theory of the free-martin. *Science* 1916;43:611–3. <https://doi.org/10.1126/science.43.1111.611>.
- [2] Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* 1945;102:400–1. <https://doi.org/10.1126/science.102.2651.400>.
- [3] Kästli F, Hall JG. Cattle twins and freemartin diagnosis. *Vet Rec* 1978;102:80–3. <https://doi.org/10.1136/vr.102.4.80>.
- [4] Padula AM. The freemartin syndrome: an update. *Anim Reprod Sci* 2005;87:93–109. <https://doi.org/10.1016/j.anireprosci.2004.09.008>.
- [5] Wijeratne WV, Munro IB, Wilkes PR. Heifer sterility associated with single-birth freemartinism. *Vet Rec* 1977;100:333–6. <https://doi.org/10.1136/vr.100.16.333>.
- [6] Murakami R-K, Miyake Y-I, Kaneda Y. Cases of XY female, single-birth freemartin and trisomy (61, XX, +20) observed in cytogenetical studies on 18 Sterile heifers. *Jpn J Vet Sci* 1989;51:941–5. <https://doi.org/10.1292/jvms1939.51.941>.
- [7] Kadokawa H, Minezawa M, Yamamoto Y, Takahashi M, Shimada K, Takahashi H, et al. Freemartinism among singleton bovine females born from multiple embryo transfer. *Theriogenology* 1995;44:295–306. [https://doi.org/10.1016/0093-691X\(95\)00179-C](https://doi.org/10.1016/0093-691X(95)00179-C).
- [8] Lindtke D, Seefried FR, Drögemüller C, Neuditschko M. Increased heterozygosity in low-pass sequencing data allows identification of blood chimeras in cattle. *Anim Genet* 2023;54:613–8. <https://doi.org/10.1111/age.13334>.
- [9] Niku M, Pessa-Morikawa T, Taponen J, Iivanainen A. Direct observation of hematopoietic progenitor chimerism in fetal freemartin cattle. *BMC Vet Res* 2007;3:29. <https://doi.org/10.1186/1746-6148-3-29>.
- [10] Vigier B, Tran D, Legeai L, Bézard J, Jossio N. Origin of Anti-Müllerian hormone in bovine freemartin fetuses. *J Reprod Fertil* 1984;70:473–9. <https://doi.org/10.1530/jrf.0.0700473>.
- [11] Shore LS, Shemesh M. Altered steroidogenesis by the fetal bovine freemartin ovary. *J Reprod Fertil* 1981;63:309–14. <https://doi.org/10.1530/jrf.0.0630309>.
- [12] Harikae K, Tsunekawa N, Hiramatsu R, Toda S, Kurohmaru M, Kanai Y. Evidence for almost complete sex-reversal in bovine freemartin gonads: formation of seminiferous tubule-like structures and transdifferentiation into typical testicular cell types. *J Reprod Dev* 2012;58:654–60. <https://doi.org/10.1262/jrd.2012-070>.
- [13] Vigier B, Locatelli A, Prépin J, Mesnil du Buisson F, Jost A. The first manifestations of freemartinism in the calf fetus do not depend on XX/XY chromosomal chimerism. *C R Acad Hebd Seances Acad Sci D* 1976;282:1355–8. PMID: 820441.
- [14] Szczerbal I, Kociucka B, Nowacka-Woszek J, Lach Z, Jaskowski JM, Switoniski M. A high incidence of leukocyte chimerism (60,XX/60,XY) in single born heifers culled due to underdevelopment of internal reproductive tracts. *Czech J Anim Sci* 2014;59:445–9. <https://doi.org/10.17221/7707-CJAS>.
- [15] Garcia-Ispuerto I, Palacín-Chauri RJ, López-Gatius F. Twin reduction in the late embryonic period prevents the condition of freemartin in dairy cattle. *Reprod Domest Anim* 2022;57:1649–51. <https://doi.org/10.1111/rda.14258>.
- [16] Maylem ERS, Dela Cruz YC, Paraguas AM, Reyes MN, Herrera JRV, Flores EB. Identifying freemartins using qualitative and quantitative PCR of the SRY gene in cattle and water Buffalo species. *CABI Agric Biosci* 2025;6:14. <https://doi.org/10.1079/ab.2025.0014>.
- [17] Kadokawa H. Original Photographic Archive for “Freemartinism among Singleton Bovine Females Born from Multiple Embryo Transfer” (Kadokawa et al., 1995). Yamaguchi University Institutional Repository; 2026. <https://petit.lib.yamaguchi-u.ac.jp/30771>. [Accessed 15 September 2026].
- [18] Lopez H, Satter LD, Wiltbank MC. Relationship between level of milk production and multiple ovulations in lactating dairy cows. *J Dairy Sci* 2005;88:2783–8. [https://doi.org/10.3168/jds.S0022-0302\(05\)72958-1](https://doi.org/10.3168/jds.S0022-0302(05)72958-1).
- [19] Cabrera VE, Fricke PM. Economics of twin pregnancies in dairy cattle. *Animals* 2021;11:552. <https://doi.org/10.3390/ani11020552>.
- [20] Agerholm JS, Buelund LE, Graff K, Sørensen TV, Secher JBM. Characteristics of unilateral and bilateral bovine twin foetuses. *Reprod Domest Anim* 2024;59:e14659. <https://doi.org/10.1111/rda.14659>.
- [21] Andreu-Vázquez C, Garcia-Ispuerto I, López-Béjar M, de Sousa NM, Beckers JF, López-Gatius F. Clinical implications of induced twin reduction in dairy cattle.

- Theriogenology 2011;76:512–21. <https://doi.org/10.1016/j.theriogenology.2011.03.003>.
- [22] Romano JE, Raudsepp T, Mulon P-Y, Villadóniga GB. Non-mosaic monosomy 59,X in cattle: a case report. *Anim Reprod Sci* 2015;156:83–90. <https://doi.org/10.1016/j.anireprosci.2015.03.004>.
- [23] Greene WA, Foote RH. Sex-chromosome ratios in cattle and their relationship to reproductive development in freemartins. *Cytogenet Cell Genet* 1977;18:97–105. <https://doi.org/10.1159/000130753>.
- [24] Sohn SH, Cho EJ, Son WJ, Lee CY. Diagnosis of bovine freemartinism by fluorescence in situ hybridization on interphase nuclei using a bovine Y chromosome-specific DNA probe. *Theriogenology* 2007;68:1003–11. <https://doi.org/10.1016/j.theriogenology.2007.06.022>.
- [25] Hirayama H, Kageyama S, Moriyasu S, Sawai K, Minamihashi A. Rapid sex chromosomal chimerism analysis in heterosexual twin female calves by loop-mediated isothermal amplification. *Anim Reprod Sci* 2007;101:38–44. <https://doi.org/10.1016/j.anireprosci.2006.09.006>.
- [26] Qiu Q, Shao T, He Y, Muhammad AUR, Cao B, Su H. Applying real-time quantitative PCR to diagnosis of freemartin in holstein cattle by quantifying SRY gene: a comparison experiment. *PeerJ* 2018;6:e4616. <https://doi.org/10.7717/peerj.4616>.
- [27] Szczerbal I, Nowacka-Woszek J, Albarella S, Switonski M. Technical note: droplet digital PCR as a new molecular method for a simple and reliable diagnosis of freemartinism in cattle. *J Dairy Sci* 2019;102:10100–4. <https://doi.org/10.3168/jds.2019-17021>.
- [28] Kozubska-Sobocińska A, Smolucha G, Danielak-Czech B. Early diagnostics of freemartinism in Polish holstein-friesian female calves. *Animals* 2019;9:971. <https://doi.org/10.3390/ani9110971>.
- [29] Rota A, Ballarin C, Vigier B, Cozzi B, Rey R. Age dependent changes in plasma Anti-Müllerian hormone concentrations in the bovine Male, female, and freemartin from birth to puberty: relationship between testosterone production and influence on sex differentiation. *Gen Comp Endocrinol* 2002;129:39–44. [https://doi.org/10.1016/S0016-6480\(02\)00514-2](https://doi.org/10.1016/S0016-6480(02)00514-2).
- [30] Remnant JG, Lea RG, Allen CE, Huxley JN, Robinson RS, Brower AI. Novel gonadal characteristics in an aged bovine freemartin. *Anim Reprod Sci* 2014;146:1–4. <https://doi.org/10.1016/j.anireprosci.2014.02.011>.