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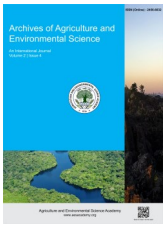

REVIEW ARTICLE

A review on molecular breeding techniques: Crucial approach in livestock improvement

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ABSTRACT

For underdeveloped countries, molecular breeding (MB) has a lot of promise. However, the implementation in developing countries is far from uniform. Livestock improvement programs aim to improve the genetics of domesticated animal populations by selecting males and females who, when mated, will produce progeny that perform better than the current generation's average. The amount of genetic progress made through conventional selection and breeding methods for quantitative traits in livestock is successful, but limitations such as routinely recording phenotypes, animal sacrifice for meat quality traits, recording in particular sex for sex-limited traits, and so on the limit the amount of genetic progress made through conventional selection and breeding methods. Marker-assisted selection (MAS), genome-wide selection (GWS), marker-assisted recurrent selection (MARS), and genome-wide sequencing (GS) are examples of modern breeding procedures. Molecular genetics technology may provide a technique to choose breeding animals at an early age (even embryos), to select for a wide variety of features and to improve the accuracy of forecasting an individual's mature phenotype. This paper examines the challenges and potential of applying molecular breeding techniques to improve livestock in developing countries.

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INTRODUCTION

Molecular breeding is the broad term used to describe various modern breeding strategies, which include marker-assisted selection (MAS), the selection of certain alleles for phenotypes conditioned by a few loci; marker-assisted backcrossing (MABC): the transfer of a limited number of loci from one genetic background to another, including transgenesis (Bernardo and Yu, 2007) Generally, livestock improvement programs aim to improve the genetics of domesticated animal populations by selecting males and females who, when mated, will produce progeny that performs better than the current generation's average (Ribaut *et al.*, 2010). Genome-wide selection (GWS) is another popular current technique: a selection based on markers without performing significance tests or selecting a subset of markers linked with the trait a priori (Bacci, 2007). Recently, marker-assisted recurrent selection (MARS) has been defined as identifying and selecting many genomic areas involved in the expression of complex phenotypes to construct the best-performing genotype within a single population or across related populations (Falconer). Since humans began to breed cattle, the goal of livestock breeding has evolved over time. The purpose of modern livestock breeding has expanded to include the development of optimal breeding measures for increasing the accuracy of breeding value estimation, shortening the generation interval, and cultivating desirable hybrids (Hayes *et al.*, 2013). The absence of well-trained workers, inadequate high-throughput capacity, poor phenotyping infrastructure, lack of information systems or suitable analysis tools, or simply resource-limited breeding programs are all contributing to the slow adoption of MB in developing nations (Dekkers, 2011). Molecular methods for animal breeding, such as marker-assisted selection (MAS) (Dekkers, 2004), genomic selection (GS) (Meuwissen *et al.*, 2001), and genome editing (Qian *et al.*,

2015; Ruan *et al*., 2015), have become increasingly important as molecular quantitative genetics techniques have advanced in recent years. Since the invention of molecular genetic technologies in the 1980s, breeders have been tasked with incorporating DNA markers into breeding theories and methods. Selective breeding based on markers of economic qualities for cattle has been established, mostly using DNA molecular marker techniques, based on major gene and quantitative trait locus (QTL) research. Many QTLs and genes linked with economic features in livestock have been identified using this approach (Dekkers, 2004). Animals have benefited from genetic advancements, particularly for features controlled by a single gene or locus. Several key genes linked to meat quality, growth, and reproduction (e.g., the porcine halothane gene and the bovine myostatin gene) have been discovered (Bertram *et al*., 2003).

In recent years, researchers have focused on genome-wide sequencing of common livestock species (Dong *et al*., 2013). Because of advancements in sequencing technology and lower costs of high-density genotyping platforms and procedures, GS can now be used in animal breeding. GS was introduced in 2001, in which the projected genomic breeding value was used to choose a suitable breeding strategy (Meuwissen *et al*., 2001). It is estimated that by employing the GS method, rates of genetic improvement in sheep and dairy cows might be boosted by 20–100% (Werf, 2013). This strategy could potentially be used to improve some immeasurable attributes. GS has been used extensively in the breeding of dairy cows. Genome editing is the most advanced technology for genetically enhancing plants and animals (Hartung and Schiemann, 2014). Genome editing has been used successfully in plant breeding, and it has a wide range of uses in precision animal breeding (Proudfoot *et al*., 2015). Furthermore, genome editing can address the drawbacks of conventional breeding, such as time-consuming multi-generational hybridization, and speeding up the breeding process (Qian *et al*., 2015). Molecular genetics is termed as the study of an individual's genetic makeup at the DNA level. It is the study of genes and genetic polymorphisms, and their identification and mapping. It is feasible to find genes involved in a range of phenotypes using molecular genetics techniques. Molecular genetics technology may provide a technique to choose breeding animals at an early age (even embryos), to select for a wide variety of features, and to improve the accuracy of forecasting an individual's mature phenotype. This paper examines the challenges and potential of applying molecular breeding techniques to improve cattle in underdeveloped countries.

Molecular genetic techniques in animal breeding: First application necessitates the integration of data from many omics' levels

The introduction of molecular genetics opened up new possibilities for improving cattle breeding programs by permitting DNA markers to identify genes or genomic areas that influence desired traits in the 1970s (Lande and Thompson, 1990). These advancements promised the identification of QTL and the development of DNA tests that could be used to select animals at an early age to aid selection decisions through marker-assisted selection (MAS), which is selection based on a combination of information derived from genetic markers associated with QTL and traditional phenotypic information (Smith and Simpson, 1986). A great number of candidate gene and QTL mapping investigations were carried out for this purpose. As a result, a large number of QTL and marker-phenotype relationships, as well as some causal mutations, were discovered (Andersson, 2001). Current livestock molecular breeding technologies, such as MAS and GS, continue at the DNA level, i.e., estimating breeding value using single-nucleotide polymorphism (SNP) sites in the genome. MAS is excellent for cattle breeding for qualities controlled by a single gene or several main genes (Soller, 1978). However, almost all the livestock's important economic qualities are complex quantitative features governed by numerous genes, making epistatic interactions difficult to forecast (Dekkers and Hospital, 2002). The accuracy of QTL placement and markers near QTLs is crucial for MAS. Furthermore, not all genetic impacts and variants can be recognized and assessed. As a result, in practical cattle breeding, only a few important genes are taken into account (Naqvi, 2007). Whole-genome resequencing has dramatically expanded the number of known variable sites in cattle genomes in recently (Li *et al*., 2013). High-throughput genotyping technologies allow for accurate and quick genome-wide genotyping on a large scale (Daetwyler *et al*., 2014). Many potential SNPs related to critical economic variables in livestock have been identified using genome-wide association analyses (Ibáñez-Escriche *et al*., 2014). Genomic-assisted breeding has substantially increased the accuracy of breeding value calculation compared to traditional breeding methods. The efficiency of GS is highly dependent on the amount of linkage disequilibrium present in a breeding population (Ai *et al*., 2015). However, because GS cannot fully explain phenotypic variances in most populations, there is tremendous room for improvement. To achieve a valid estimate of breeding value when performing GS, a reference group must be formed. In other unrelated populations, however, effectively confirming the estimate using the reference group is problematic (Meuwissen *et al*., 2001). Furthermore, GS ignores the impact of variation other than the genome on phenotypic variance (e.g., methylation, mRNA, and non-coding RNA expression).

Molecular breeding methods vs. traditional breeding methods

- In theory, early access to molecular genetic information as early as the embryo stage allows for early selection and reduced generation intervals.
- If there are no genotyping errors, molecular genetic information is unaffected by environmental factors and so has a heritability of 1.
- All selection candidates can get molecular genetic information, which is especially useful for sex-limited qualities, features that are expensive or difficult to record, or traits that require the animal to be slaughtered (carcass traits).
- We may select wide range of qualities using a molecular approach, saving time and effort.

- Molecular genetic information improves the accuracy of forecasting an individual's mature phenotype.

Identification of Genetic Markers in MAS (Marker-Assisted Selection): a major tool for livestock improvements via animal breeding

Marker-Assisted Selection (MAS) combines genetic and phenotypic data to improve the selection response to a standard technique. MAS is an indirect selection method in which a trait of interest is chosen based on a marker associated with it rather than the trait itself (Ribaut and Ragot, 2007). The goal is to improve genetic evaluation and selection by combining all genetic information at markers and QTL with phenotypic data (Atienza *et al*., 2003).The advantage of employing MAS is that the effect of genes on production may be measured directly on the genetic composition of the animal rather than being estimated from the phenotype (Naqvi, 2007). wo selection procedures, namely classic or conventional selection methods and molecular genetics methods, are used to improve the selection response. Multiple estimated QTL effects and multiple trait selection may aid in making better decisions about the application of MAS in animal improvement (Neeteson *et al*., 1999). The extra genetic gain due to MAS decreases very quickly with the number of generations of selection for the same QTL, and the identification rate of new QTL is difficult to predict. The gain due to MAS for a specific QTL is higher when the characteristic like fertility and carcass is measured after the selection. MAS aims is to improve selection response (Ron and Weller, 2007). Identification of specific polymorphisms responsible for the observed effect is required for successfully deploy of such QTL inside selection processes (Franklin and Mayo, 1998). MAS's efficacy is determined by recombination between the marker and the real QTL, as well as mutation elsewhere in the genome (Keightley and Hill, 1992). In domestic animals, both candidate gene and QTL (quantitative trait loci) mapping methodologies have been frequently used in genome wide association studies (GWAS) to uncover genetic markers acceptable for MAS (Fan *et al*., 2010). MAS can result in genetic gains of 10-20%, depending on the size of the QTL. In comparison to conventional selection based on BLUP , when MAS is utilized in a population, the frequency of the beneficial QTL allele increases swiftly during the first generations (Best linear unbiased prediction) (Rothschild *et al*., 2007). The best candidates for being the parents of the next generation should be identified. MAS can be employed effectively for traits governed by a QTL with significant effects and for which phenotypic selection is expensive. The usage of MAS, on the other hand, necessitates linkage disequilibrium, which might be exploited in dairy cattle as MAS within the family. The enormous number of offspring necessary from each half-sib family to evaluate unbiased effects is one issue with MAS within the family. Dairy cattle selection techniques based on marker information were mostly dependent on information from within families (Spelman and Bovenhuis, 1998). After ine-mapping QTL, the next step is to use them to predict breeding values. Here are a few examples of LD markers being used to pre-select candidates in dairy cattle (Uleberg and Meuwissen, 2007). MAS has proven to be a useful method in selecting organisms for desirable features when used in conjunction with standard selection strategies. By selecting promising young bulls early, MAS is intended to maximize genetic gain and lower the cost of progeny testing compared to traditional breeding programs. The ability of breeders to spend on genotypic information that helps them improve their commercial breeding activities is dependent on their knowledge of variable marker information from animal to animal and the different effects on numerous traits. MAS also appears to be a viable method for selecting animals with genetic disease resistance. The development of large-scale genotyping methods and infrastructure that allows the generation of hundreds of thousands of molecular data at a reasonable cost will be required in the future to make MAS effective in large breeding populations.

Role of assisted reproductive technologies in livestock improvement

The intensity of genetic improvement and profitability are determined by the animal's reproductive performance. An animal's reproductive performance is influenced by genetic improvements on either the male or female side, or both. Various reproductive methods have been created and modified to overcome the economic losses in animal production caused by reproductive inefficiency. Cattle, together with tiny ruminants, now make up the majority of the economy of large, medium, and small farms in developing countries around the world, and in most cases, they are the most valuable economic asset in terms of milk, meat, and wool production (Rodriguez-Martinez, 2012). Following the enormous flow of information around us and the increasing global commercial interests in areas where cattle production has its major assets, commercialization of animal biotechnologies, including those related to reproduction [also known as assisted reproductive techniques (ARTS)], is becoming a reality in developing countries (Faber *et al*., 2003). Reproductive biotechnologies will be employed on a regular basis to decrease generational gaps and spread genetic material among breeding animal populations. Artificial insemination (AI), embryo transfer (ET), manipulation of fertilization and embryo development in vitro (IVF), and multiplication techniques (cloning) for the application of transgenesis have all been developed over generations to attain this goal (M Morrell and Rodriguez-Martinez, 2009). Artificial insemination, in vitro production, superovulation, embryo transfer, transgenesis, and cloning are all examples of advances in assisted reproductive technologies (ART). These procedures were first developed to address reproductive issues, but they have had a considerable impact on animal breeding (Gelayenew and Asebe, 2016). All of these technologies are capable of speeding up genetic alterations by reducing generation intervals and boosting selection program accuracy (Khare and Khare, 2017). Artificial insemination (AI) and embryo transfer (ET) are two of the most well-known methods used in livestock production in both industrialized and developing countries (Smidt and Niemann, 1999).

Transgenic animals and cloning are two recent advancements in biotechnology technologies for reproduction (Kahi and Rewe, 2008). Because it enhances the rate of reproduction and decreases the generation time, RT will have a long-term impact on animal breeding (Wajid *et al.*, 2013) . The most effective reproductive technologies, such as AI and ET, necessitated the use of several emerging biotechnologies, such as Multiple Ovulation and Embryo Transfer (MOET), In Vitro Fertilization (IVF), and cloning, to a great extent (Rahman *et al.*, 2008).

Artificial insemination: Artificial insemination is a process in which a male's sperm is collected and manually injected into the female reproductive canal at the appropriate time using a method other than natural mating (Vishwanath, 2003). Artificial intelligence has been employed in most domestic creatures, including bees, as well as in humans. In livestock, it is the most widely used ART (Knox, 2016). AI has made a significant contribution to genetic improvement and disease control (Thibier and Wagner, 2002). This technology maximizes the use of high breeding value males, the dissemination of superior genetic materials, the introduction of new genetic material through semen importation, the use of frozen semen even after the donor is dead or the animal is physically unable to mount and reduces the risk of sexually transmitted disease transmission (Salisbury and VanDemark, 1961). The use of sex-sorted sperm for AI has been touted as a technique of enhancing animal reproductive efficiency, particularly in the dairy industry, where males have less commercial value. Every year, about 100 million cattle, 0.5 million goats, 3.3 million sheep, 40 million pigs, and 0.5 million goats are artificially inseminated (Ashebir *et al.*, 2016).

Estrous synchronization: Estrus synchronization is the manipulation of an animal's natural reproductive cycle to have as many females at the same time as possible in order to reduce the breeding period (Abdullah *et al.*, 2008). The use of a synchronization program based on the combination of gonadotrophin-releasing hormone (GnRH) with prostaglandin F2α (PGF2α)) and progesterone is common (Whitley and Jackson, 2004). Major reproductive difficulties such as anestrus, recurrent breeding, and delayed estrus can be resolved using various estrus synchronization protocols. Estrus synchronization can be accomplished in two ways:

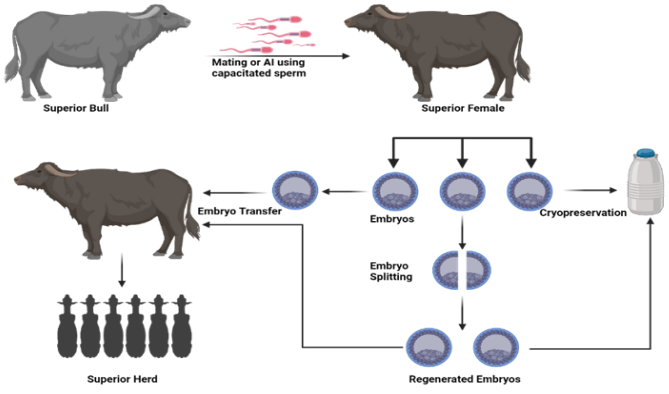

Figure 1. *Schematic Presentation of Multiple Ovulation Embryo Transfer in Buffalo.*

I. Non-hormonal methods include the use of plant-derived heat/estrus inducers such as Prajana, Fertivet, as well as mineral supplements and ovarian massage.

II. Hormonal accomplish use of Estrogen, GnRH, Progesterone, Prostaglandins, and Insulin as hormonal factors. Depending on the animal's species and ovarian stage, different hormonal synchronization protocols are used.

- **PGF2α:** In cyclic females, two types of PGF2α should be given at 0 and 11 days apart. Estrus detection is usually not required before or after injections. Regardless of what stage of the estrous cycle they were in when the first injection was given, all cyclic animals will respond to the second injection (Ataman and Aköz, 2006).
- **GnRH-PGF2α-GnRH Protocol:** The GnRH-PGF2α-GnRH treatment can be used on cyclic animals on any day of their oestrous cycle. This protocol calls for a GnRH injection on day 1, followed by a PGF2α injection on day 8, then another GnRH injection on day 10. On day 11, insemination is recommended. This program's benefit is that it promotes oestrus in non-cyclic females who are at least 30 days postpartum (Ataman and Aköz, 2006).
- **CIDR:** The basic approach entails implanting the CIDR (Controlled Internal Drug Release-Progestin Impregnated Plastic Devices) into the vagina for seven days, administering a PGF2α injection on day six of implantation, and observing oestrus on day eight (Macmillan and Peterson, 1993).
- **Multiple ovulation embryo transfer:** MOET is a technique in which many eggs are fertilized in an animal and the embryo is extracted on the seventh day, usually at the blastocyst stage, non-surgically (flushing). After grading, the resulting embryo can be transferred to a synchronized recipient or frozen for future use shown in Figure 1. Follicular stimulating hormone (FSH) is used to cause the ovary to release more than one egg in a cycle, which aids in the treatment of high-breed animals with a low production rate or poor success rate (Menchaca *et al.*, 2009). The great heterogeneity in the ovulatory response to hormone treatment, as well as the low and variable number of transferrable embryos and offspring obtained, continue to limit the utilization of MOET treatments (Tobă *et al.*, 2012).
- **In-vitro embryo production:** It necessitates the precise manipulation of oocyte collection and maturation (IVM), fertilization (IVF), and presumptive zygotes culture (IVC) at a certain stage (usually blastocyst stage) (Gilchrist, 2010). Embryos can then be frozen or transferred to estrous synchronized recipients. IVP is a biotechnique that is utilized commercially, in basic research, and in the treatment of infertility (Nogueira *et al.*, 2012). Laparotomy (in all species), laparoscopy (in sheep, goats, and swine, as well as horses and cattle), and ovum pick up (OPU) are used to collect oocytes from live animals (cattle, horses) (Nogueira *et al.*, 2012).

- In vitro embryo creation is a procedure that can be used instead of insemination or a multiple ovulation and embryo transfer (MOET) program. Oocyte donors might be immature or pregnant females, as well as immunologically sterile females. This method allows for embryonic and somatic cloning, as well as the creation of transgenic humans, their cloning, and chimeras (Smith, 1988). The success of invitro manufacturing is due to a number of factors:

I. Sperm and oocyte source: sperm and oocyte donor animal's age and physiological status.
II. Important technical factors: maturation media, fertilization, and culture.
III. Temperature, CO_2 concentration, and humidity (Camargo *et al.*, 2018).

Despite the benefits of IVP, invitro-generated embryos have a lower cryopreservation survival rate than embryos produced in viv (Thibier, 2011). Though the underlying cause of enhanced cryo-sensitivity is unknown, one of the causes is the buildup of higher intracellular lipid in invitro-produced embryos (Ferré *et al.*, 2020)

Intra-cytoplasmic Sperm Injection (ICSI): ICSI is a micromanipulation method in which a single spermatozoon is injected into the cytoplasm of a mature egg. For the development of kids from oligospermic males or individuals with a high degree of sperm abnormalities, ICSI can be useful (Briski and Salamone, 2022). It is used to treat male infertility in animals, as well as circumstances where sperm cannot easily access eggs. When all other assisted reproductive procedures have failed, ICSI is indicated (Parmar *et al.*, 2013). Intracytoplasmic sperm injection could thus be employed in livestock species for a variety of purposes, including biodiversity conservation, transgenic production, and resolving fertilization issues in IVF systems represented in Figure 2 (Horiuchi and Numabe, 1999). Due to the limitation of polyspermy in pigs, despite improvements in IVP. The ICSI technique can be used to circumvent a cryopreserved egg that has a polyspermy problem due to early cortical granule ejection (Salamone *et al.*, 2017).

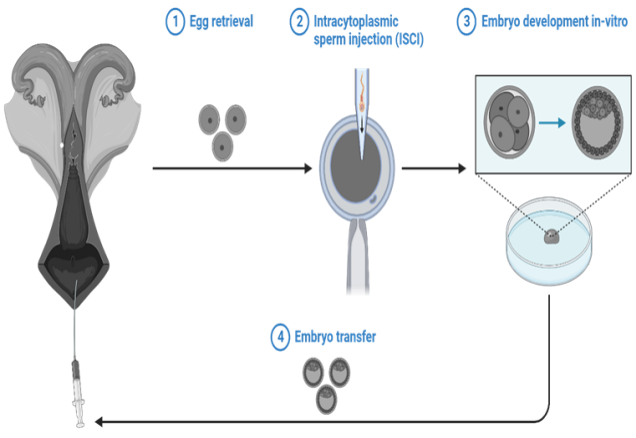

Figure 2. *Concept of intra-cytoplasmic sperm injection.*

Cloning: Cloning is the process of naturally or artificially creating genetically identical individuals of an organism (Gurdon and Colman, 1999). It is a powerful strategy that, more importantly, might be used to multiply elite individuals and reduce genetic variation in experimental animals. This method can be used for both conservation and reproduction of endangered species (Holt *et al.*, 2004). It can be employed for therapeutic cloning and the creation of stem cells for therapeutic purposes. Cloning with somatic cells allows researchers to select and generate animals with specific characteristics (Ryder, 2002). The first animal obtained through somatic cloning was a sheep named Dolly (García-Sancho, 2015). The procedure for cloning a specific agricultural animal using SCNT is same for all animals (Campbell *et al.*, 2007). The somatic cells from the animal that will be cloned are collected initially. The somatic cells could be employed right away or kept in the lab for future use (Niemann and Lucas-Hahn, 2012). The most difficult element of SCNT is extracting maternal DNA from a metaphase II egg. After that, the somatic nucleus can be introduced into the cytoplasm of an egg. This results in a single-cell embryo (Ogura *et al.*, 2013). The electrical current is then applied to the grouped somatic cell and egg cytoplasm. With this energy, the cloned embryo should be able to start developing. Surrogate recipients, such as a cow or sheep in the case of farm animals, are subsequently used to implant the successfully grown embryos (Edwards *et al.*, 2003). Cloning samples from various species for the conservation of accessible genetic variety can be done in areas where sampling and storage of adequate samples of semen and embryos is not possible (Brown and Marshall, 1995). Local breeds with valuable genes, particularly for heat tolerance or disease resistance, must be saved from extinction through cloning techniques (Verma *et al.*, 2012). Cloning may be employed in xenotransplantation in the future since it allows for the proliferation of humanized pigs whose organs can be transplanted into humans (Schmidt *et al.*, 2002).

Transgenesis and its application in livestock improvement

Transgenesis is the process of inserting a foreign gene (transgene) into an organism's genome in the hopes that the ensuing transgenic organism would express the gene and display a new attribute or characteristic (Kind and Schnieke, 2008). Transgenic animals and genetically modified organisms (GMOs) are organisms that have had a portion of foreign DNA inserted into their genome, or that have had any alteration to their genome sequence introduced intentionally (Meng *et al.*, 1998). Gene transfer could speed up and facilitate genetic progress. The use of transgenic animal technology is altering the way we domesticate livestock (Wall *et al.*, 1997). Transgenesis is the process of introducing a foreign gene (of interest) into the genome of another species in such a way that it is handed down from generation to generation (Goldman *et al.*, 2004). The process of creating transgenic animals began with the goal of creating better breed lines that are stronger, have more carcass, have a faster growth rate, and produce more milk (Duszewska *et al.*, 2010). Transgenic animals are bred to improve qualitative and quantitative qualities in cattle while also reducing disease

susceptibility (Gelayenew and Asebe, 2016). This approach involves cloning a transgene encoding a specific phenotype into a vector, which could be synthetic, viral, or plasmid DNA, and inserting the hybrid vector into the host organism's genome (Babar *et al.*, 2013). To make transgenic animals, a variety of ways have been devised, some of which have had a lot of success and others which are still being studied. The microinjection of a transgene into the pronucleus of a newly fertilized egg, the introduction of a desired gene into embryonic stem cells, and the transgenic somatic cell nuclear transfer (TSCNT), which is a variant of SCNT, are the most frequent ways for creating transgenic animals (Wajid *et al.*, 2013).

Pronuclear microinjection: Microinjection of foreign DNA into the pronucleus of a recently fertilized egg (zygote) is the most common approach known (Wall, 2001). The process entails inserting DNA carrying the desired genes into the male pronucleus of zygotes under the control of an appropriate promoter, followed by the transfer of embryos to a surrogate mother (Co *et al.*, 2000). The transgene finally integrates in an ad hoc manner into the embryonic DNA. Goats, rabbits, pigs, and sheep are all good candidates for this operation. However, due to difficulty in visualizing the male pronuclear in certain species, such as cattle, it produces unsatisfactory results (Niemann *et al.*, 2005). The success rate of transgenic integration in rats, mice, and rabbits ranges from 3% to greater than 1% in pigs, sheep, and cows (Rülicke and Hübscher, 2000). The determination of transgene integration is a limitation. The only way to detect transgene integration is to examine the transgenic animal and its offspring, which can be difficult because reproductive (including the time before reaching physiological maturity) is long in large animals, ranging from 1.0–2.3 years in pigs, 0.9–2.3 years in goats, and 2.3–4.5 years in cows (Wall and Seidel Jr, 1992). Furthermore, due to the high degree of mosaicism, transgenic animals created using this approach have a wide range of transgene expression (Chan *et al.*, 1999). Testing numerous lines of animals for optimal transgene expression is essential to develop transgenic animals, which is a time-consuming and expensive process (Meyer, 1995). In the case of large animal transgenesis, there is a significant disadvantage. In the case of small animals like mice, rats, and rabbits, however, the approaches are frequently used to create transgenic animals (Giacomotto and Ségalat, 2010).

Sperm Mediated Gene Transfer (SMGT): Sperm mediated gene transfer (SMGT) is a technology that uses spermatozoa's inherent capacity to transfer foreign DNA into the egg during fertilization (Bacci, 2007). It is based on sperm cells' inherent ability to bind and internalize foreign DNA molecules before transferring them to the egg during fertilization (Lavitrano *et al.*, 2005). There are numerous studies on the use of sperm cells as a vector containing transgene as an alternative to traditional pronuclear microinjection (Lavitrano *et al.*, 1997). It was discovered in 1971 that sperm cells have the ability to transfer foreign DNA into the egg during fertilization (Brackett *et al.*, 1971). The beauty of SMGT is that it transports exogenous DNA using a "natural" genetic material carrier, namely the sperm cell. In the subacrosomal region and near the equatorial area, foreign DNA molecules bind to the sperm cell's head. DNA molecules are taken up by the cell membrane once they are attached to it (Francolini *et al.*, 1993). The sperm cells are first co-incubated with the transgenic so that the foreign DNA bearing gene of interest is taken up by the sperm cells. DNA adheres to the plasma membrane of sperm cells thanks to a DNA-binding protein found in sperm (Pereyra-Bonnet *et al.*, 2010). In vitro fertilization (IVF), laparoscopic insemination (LI), and intracytoplasmic sperm injection are among the several techniques (ICSI) (Pereyra-Bonnet *et al.*, 2010). Even though IVF and LI produce more embryos than ICSI, it has been stated that ICSI is a better approach for the generation of transgenic animals (Pajooh and Tajik, 2020). Sperm-mediated gene transfer could also be used to create multigene transgenic pigs that could be useful as large animal models for medical research, agricultural and pharmaceutical applications, and, in particular, xenotransplantation, which requires extensive genetic manipulation of donor pigs to make them suitable for human grafting (Smith and Spadafora, 2005).

Somatic Cell Nuclear Transfer (SCNT): The geneticist's toolbox includes somatic cell nuclear transfer, which involves transferring a donor cell from one person into an enucleated, unfertilized egg from another (Baguisi *et al.*, 1999). This method has been investigated as a potential tool for breeding exceptional cattle, protecting endangered species, and biomedical research (Kishigami *et al.*, 2008). As a result, mastering SCNT and other ARTs in dogs will have various implications for human and veterinary medicine (Wani *et al.*, 2010). The nucleus of a somatic cell is transferred to the cytoplasm of enucleated oocytes in SCNT to create a genetically identical replica of the nuclear donor (Jang *et al.*, 2006). SCNT cloning has resulted in the generation of transgenic animals for agricultural and biomedical purposes, as well as the production of animals with valuable features (Lee *et al.*, 2020). SCNT has been used to successfully produce cloned and transgenic cloned animals (Kurome *et al.*, 2007). The success rate of SCNT in most animals, including cattle, is between 1% and 3%. SCNT, on the other hand, has serious issues with low efficiency and high fetal and embryo mortality rates (Lagutina *et al.*, 2007). SCNT efficiency is influenced by several parameters, including the donor cell type, electro-fusion, and activation processes (Liu *et al.*, 2007).

Embryonic stem cells-mediated transgenesis: ES cells are pluripotent stem cells derived from the blastocyst's inner cell mass, and they're commonly employed to make transgenic mice (Kubota and Brinster, 2006). These cells can divide indefinitely under ideal cultivation conditions. Because of this property, ES cells may be reproduced quickly and easily manipulated by inserting a DNA construct encoding genes of interest (Prelle *et al.*, 2002). The procedure entails isolating and cultivating ES cells in vitro before inserting the transgene. The transgenic ES cells are then separated from the non-transgenic cells and allowed to multiply (Yeom *et al.*, 1996). The transgenic ES cells are then

chosen, injected into blastocysts, and then transferred to the surrogate mother to produce transgenic ES cell colonies (Wakayama *et al.*, 2001). The germline transmission of the chimeric animals is tested, and pure transgenic animals are created using various breeding procedures (Miao, 2013). ES cells were previously only used in mice as a means of transgenesis, however, attempts to isolate Embryonic Stem cells in farm animals have also been made (Mehta *et al.*, 2017). However, in these species, this approach fails due to demanding culture conditions, the need to maintain a culture in an undifferentiated state, and the need for complicated genetic manipulation. Even though cattle ES cells were compared to mouse ES cells, the long generation time and high expense of maintaining numerous chimera animals make germline transfer testing difficult (Brevini *et al.*, 2008).

Importance of GENE Editing and its application in livestock improvement

Despite the fact that the world's population is estimated to be 7.6 billion people, one out of every nine of us (821 million people) does not have enough food to live a normal, active life. Despite the challenges of feeding our species, the human population is expected to rise to 8.5 billion by 2030, 9.7 billion by 2050, and 11.2 billion by 2100 (Tait-Burkard *et al.*, 2018). Clearly, if we are already struggling to feed 7.5 billion people, preparing to feed nearly 4 billion more people will be one of our species' greatest problems. Different types problem are presented at this situation which are very difficult to cure or takes long term and become very economic. To overcome problem like this numerous genomic editing tools and techniques are developed to compensate the demand for livestock improvement. With the help of gene editing technology many researchers can change the genes in the genomes of animals according to their desires. This latest trend new technology for livestock improvement. Gene editing techniques like Zinc Finger Nuclease (ZFN), Transcription Activator Like Effector Nuclease (TALEN) and Cluster Regulatory Interspaced Short Palindromic Repeats (CRISPR) are important tools for livestock genetic change and remodeling in future generations (Gaj *et al.*, 2013). All of these nucleases act by causing site-specific DNA double-strand breaks (DSBs) at specific locations in the genome, which are then "patched" up by the cell's repair system using either the non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. NHEJ is a significant DNA repair process in cells that directly ligates break ends without the use of a template. Nucleotide insertions or deletions occur during the NHEJ repair process, resulting in indels and repair error-related frameshift. As a result, nuclease-assisted NHEJ can be used to knock off genes efficiently. HDR, on the other hand, occurs at a lower frequency in cells than NHEJ, but it is accomplished through homologous recombination between a donor DNA template and the target genomic locus, resulting in a repair (Christian *et al.*, 2010; Doyon *et al.*, 2008). ZFNs, which consists of a zinc finger DNA-binding domain and a FokI DNA cleavage domain, were modified in 2007 by Sangamo Biosciences Inc., which lowered the off-target rate (Miller *et al.*, 2007). Sangamo then successfully carried out ZFN-mediated gene deletion in zebrafish (Doyon *et al.*, 2008). The editing efficiency reached 20%, which was 200 times better than traditional targeting efficiency (*0.1%). Genome editing technology drew widespread attention and was quickly used to study in zebrafish, humans, mice, swine, cattle, and other species (Liu *et al.*, 2013; Yang *et al.*, 2011). However, due to expensive prices, technical problems, and the long time it takes to implement this technology in conventional labs, it is extremely difficult to do so. In 2010, the Voytas lab constructed artificial TALENs using naturally occurring transcription activator-like effector (TALE) repeat arrays fused with FokI (a nuclease isolated from the bacterium Flavobacterium okeanokoites) and confirmed that TALENs performed targeted cleavage of target DNA (Christian *et al.*, 2010). TALENs had a simpler preparation technique than ZFNs, and they had more target selection choices as well. The employment of genome editing technologies in conventional research labs was made possible by TALENs, paving the way for a new front in the genome editing revolution. However, in February 2013, researchers from the Massachusetts Institute of Technology and Harvard University employed CRISPR enzymes from Streptococcus pyogenes and Streptococcus thermophiles, as well as synthetic RNA, to modify the genomes of mouse and human cells (Cong *et al.*, 2013). These researchers demonstrated the use of the CRISPR/Cas9 system in genome editing in mammalian cells for the first time. The CRISPR/Cas9 gene-editing technology, such as Cas9/gRNA Ribonucleoproteins (RNPs), was significantly easier to build and operate than the ZFN and TALEN technologies. Only the Cas9 protein and a single guide RNA make up the entire mechanism (sgRNA). The vector may be constructed in a week and has a high gene editing efficiency (up to 100 percent in certain cases) (Mali *et al.*, 2013). CRISPR/Cas9 can also be multiplexed, which means it can target many genomic loci at the same time. Furthermore, it has been proved to work in all species that have been examined thus far. The CRISPR/Cas9 method is reliable, efficient, and inexpensive, and it has propelled genome editing to new heights. Today, almost every lab can conduct genome editing experiments, covering any species of interest (Ruan *et al.*, 2017).

Gene editing techniques

Zinc Finger Nucleases (ZFN): ZFNs or chimeric nucleases, were created in 2001 with the goal of targeting and disrupting specific DNA sequences (Qomi *et al.*, 2019). Zinc fingers are tiny protein motifs (20–30 amino acids) that are controlled by the zinc ion, which binds to DNA and recognizes a 3-base pair (bp) pattern. The motifs were paired with the genetically modified restriction enzyme FokI to make a programmable nuclease that can recognize target sequence locations. When two zinc finger modules attach to DNA in opposing locations with the FokI enzyme in the middle, forming a homodimer complex, the ZFNs are effective. The nuclease breaks both DNA strands once homo -dimerization is established, and mutations are randomly introduced (Adli, 2018). The target site can be constructed by altering the residues in a single zinc finger (Figure 3), which

changes its specificity for DNA recognition, allowing the finger motifs to identify a wide range of DNA triplet nucleotides (Carroll, 2017). ZFNs were revolutionary because of their increased specificity to DNA sequences, but they have a few drawbacks, such as the time-consuming procedure of designing a pair of ZFNs against a target sequence. Furthermore, the genome has a limited number of possible targets, making this gene editing tool unsuitable for many investigations. In fact, only one locus per 50 bp is acceptable for this method (Qomi *et al.*, 2019).

Transcriptional Activator-Like Effector Nucleases (TALENs): The TALEN system (Bedell *et al.*, 2012) is a widely used approach for precise genome editing that has been in use for several years. The FokI cleavage domain was combined with the DNA-binding domains of TALE proteins to create TALENs. For the efficient edition of a single base pair, TALEs are made up of multiplex repeats of 34 amino acids (Zhang *et al.*, 2019). TALENs, like ZFNs, stimulate targeted DSBs that aid in the initiation of DNA damage pathways and assure changes. A core domain responsible for DNA binding and a nuclear localization sequence are two proteins involved in the TALEN system (Schornack *et al.*, 2006). For the first time in 2007, these proteins were discovered to have the ability to bind to DNA. The DNA-binding domain, on the other hand, has a 34-amino-acid repeating sequence, each of which perceives a single nucleotide in the target DNA, whereas ZFNs detect three nucleotides in the target DNA (Römer *et al.*, 2007). The number of research using ZFNs and TALENs in plants is few, and these findings appear to prefer TALENs; nonetheless, the editing efficiency of these two nucleases is rather low. As a result, the use of TALENs is more unaffected and programming-friendly. TALE targets are identified by the presence of repeat variable di-residue (RVD) flanked at positions 12 and 13 of each target sequence (Bedell *et al.*, 2012; Gaj *et al.*, 2013) DNA repetitive sequences can affect TALE proteins in general. TALE proteins always fix the nucleotides of the DNA sequence at the 50th thymidine base, according to previous study. In the absence of a 50T, TALE transcription factors (TALE-TFs) and TALE recombinase (TALE-R) activity is diminished (Lamb *et al.*, 2013). Because their modulation is much easier and less expensive, and their o- target rate is significantly lower, TALENs are preferred over ZFNs.

CRISPR/Cas9 system: CRISPR/Cas9 is a third-generation gene editing method that uses a bacterium or archaea's intentionally created an immune system to mediate foreign DNA breakdown and so function as a defense mechanism against viral infections (Mojica *et al.*, 2005). CRISPR (clustered regularly interspaced short palindromic repeats) were first discovered in the genome of E. coli in 1987, but it wasn't until 2012 that CRISPR and the CRISPR associated protein Cas9 were reported to cut DNA duplex at particular places in vitro (Jinek *et al.*, 2012). This discovery bolstered the use of CRISPR/Cas9 in gene editing. (Cong *et al.*, 2013) established two CRISPR/Cas9 systems and demonstrated that short RNAs could instruct Cas9 nucleases to induce precise cleavage at endogenous genomic loci in human and mouse cells a year later. This is a significant development in the field of gene editing (Wei *et al.*, 2015). CRISPR consists of a leader sequence, numerous repeat sequences, and several spacer sequences. The CRISPR gene cluster is positioned upstream of the leader sequence. It has no coding activity but acts as a promoter for a certain species. The repeat sequences are palindromic sequences with high conservation that can form hairpin structures. Spacer sequences disrupt the repeat sequences, which are not organized in tandem shown in Figure 4. The spacer sequences are identical to several regions found in the genomes of phages and plasmids, allowing cells to recognize and defend against these phages or plasmids (Stern *et al.*, 2010). Cas9 nuclease complexes are made up of the Cas9 protein, a CRISPR RNA (crRNA), and a transactivation CRISPR RNA (tracrRNA). The crRNA and tracrRNA bind to the Cas9 protein as a dimer. The complex identifies and slices target DNA at precise locations, resulting in double-strand breaks that cause cells to repair their DNA. Repair occurs by NHEJ in the absence of homologous DNA, making gene deletion easier (Takasu *et al.*, 2016). When homologous DNA is present, HR occurs, allowing for easier gene insertion.

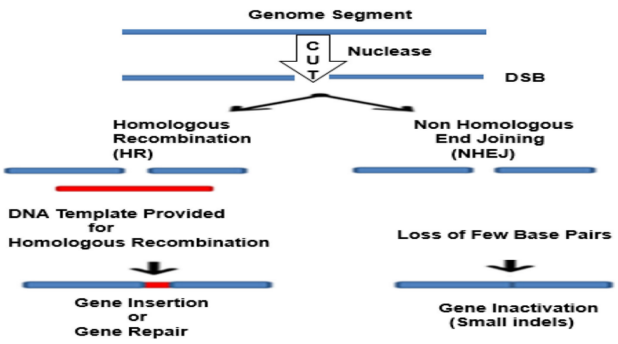

Figure 3. *Genome editing outcomes. Genome editing nucleases induce double-strand breaks (DSBs). The breaks are repaired through two ways: by non-homologous end joining (NHEJ) in the absence of a donor template or via homologous recombination (HR) in the presence of a donor template. The NHEJ creates few base insertions or deletion, resulting in an indel,or in frameshift that causes gene disruption. In the HR pathway, a donor DNA (a plasmid or single-stranded oligonucleotide) can be integrated to the target site to modify the gene, introducing the nucleotides and leading to insertion of cDNA or frameshifts induction (Adapted from Christian et al., 2010)*

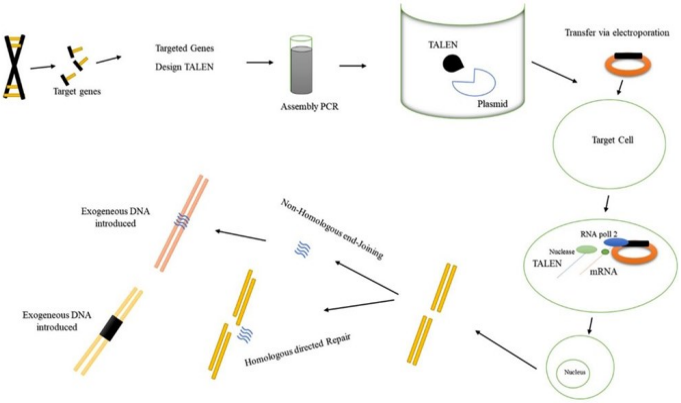

Figure 4. *Transcription activator-like e_ector nucleases (TALENs) are dimeric transcription factors/nucleases engineered from an array of 34-amino-acid molecules, each of which targets one nucleotide. The target sequence is recognized; a corresponding TALEN sequence is built and insertedinto a cellular plasmid. The cellular plasmid is inserted into the host cell, where it is translated to produce the functional TALEN, which penetrates the nucleus and binds to and cleaves the target sequence. The applications of this system include the knockout of a target gene or the addition of a replacement nucleotide into the target gene.*

Advantages and disadvantages of different gene editing techniques

In comparison to standard HR, ZFN technique offers a much higher gene targeting efficiency (10–30%). The ZFN approach does not require embryonic stem cells and can be used on a wide range of eukaryotic cells. ZFN is the most well-known of the first -generation gene editing techniques. The ZFN recognition domain, on the other hand, is context-dependent. Its constituent amino acid repeatedly interact with one another, lowering gene targeting specificity and efficiency (Sander *et al.*, 2011). In reality, designing a good ZFN for any target gene is tough. To put it another way, ZFN can't alter every gene in the genome. Furthermore, the ZFN method has off-target effects, resulting in cytotoxicity (Sung *et al.*, 2014). Among the three approaches, the CRISPR/Cas9 system has the highest targeting efficiency (50–80%) (Fu *et al.*, 2013). The creation of an RNA sequence that is complementary to the target DNA sequence is fundamental to the formation of the CRISPR/Cas9 system. CRISPR/Cas9 is less expensive and easier to use than ZFN and TALEN for target site design, vector building, and operation. Both ZFN and TALEN are a kind of TALEN represented in Figure 5.

Proteins target DNA, whereas the CRISPR/Cas9 system uses RNA to target DNA through a base-pairing mechanism. As a result, the CRISPR/Cas9 system recognizes DNA more accurately, with less off-target effects and decreased cytotoxicity. This technology, on the other hand, was invented considerably later and is currently in development. Additional mutations at sites other than the target site have been reported (Ding *et al.*, 2013). Cutting of the target sequence is also dependent on a few short PAMs in addition to the matching crRNA sequence. If there is no PAM around the target sequence, the Cas9 protein does not cut it.

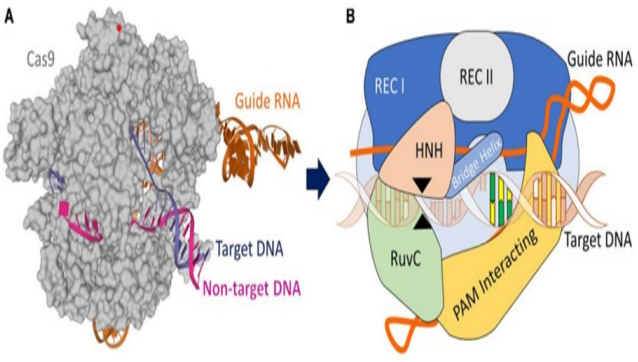

Figure 5. *CRISPR/Cas9 structure. (A) X-ray structure of the Streptococcus pyogenes (Sp) CRISPR/Cas9 system (5F9R.pdb) in the pre-activated state (Jiang et al., 2016), created using Mol* (Sehnal et al., 2018). Cas9(gray) is shown in molecular surface. The guide RNA (orange), the target DNA (dark blue), and non-target DNA (pink) strands are shown as cartoons. (B) A schematic CRISPR/Cas9 ribonucleoprotein structure formedby six domains: Rec I, Rec II, RuvC, HNH, Bridge Helix, and PAM Interacting domain, and guide RNA targeting DNA. The black arrow heads indicate the cut sites from each RuvC and HNH domains. The yellow/green nucleotides represent the PAM sequence.*

Applications of gene editing techniques in animal husbandry

Increases the production of livestock products

MSTN (myostatin) is a protein that inhibits muscle growth and development. The "double- muscle" characteristic is caused by the loss of MSTN function. Qian *et al.* (2015) altered the MSTN gene in fibroblasts obtained from Meishan pig fetuses using the ZFN method. MSTN-mutated Meishan pigs were created by somatic cell nuclear transplantation. MSTN mutant Meishan pigs produce 11.62 percent more lean meat than wild-type pigs (Qian *et al.*, 2015). Yu *et al.* (2016) also created 272 monoclonal cells with MSTN gene mutations by designing and transfecting a pair of TALENs into goat fibroblasts. Ten clones with diverse genotypes were utilized as donors for somatic cell nuclear transplantation, and three goat clones were obtained (K179/MSTN/, K52-2/MSTN+/, as well as K52-1/MSTN+/+). Meat yield was considerably higher in the goat with the mutant MSTN gene. (Yu *et al.*, 2016) found that the double knockout goat clone (K179/MSTN/) had 1.7-fold higher body weight than the wild- type (K52-1/MSTN+/+), while the single knockout goat clone (K179/MSTN+/) was 32 percent heavier than the wild-type (Proudfoot *et al.*, 2015). In addition, gene editing techniques have been used to create MSTN mutant animals in some other farm animal species with the goal of promoting muscle development or increasing meat production (Wang *et al.*, 2016). Hair growth is regulated by the regulatory factor fibroblast growth factor 5 (FGF-5). It causes hair follicles to transition from the growing to the recessive phase. The FGF5 gene mutation causes the growth phase to be prolonged, resulting in increased hair length. Microinjected MSTN- and FGF5-targeting vector Cas9 mRNA and sgRNAs into cashmere goat embryos using the CRISPR/Cas9 technique and analyzed the top three economic features in cashmere goats, including cashmere yield, cashmere fiber diameter, and length, using the CRISPR/Cas9 technique. The GFG5 mutant cashmere goats had much longer cashmere fibers and larger cashmere yields, according to the findings (Wang *et al.*, 2016).

Improves animal fertility and disease resistance

Porcine reproductive and respiratory syndrome (PRRS), often known as blue-ear pig illness, is a difficult condition for pig producers all over the world. In young pigs, it causes severe respiratory issues, as well as breeding failure in pregnant females. It also causes abortions, premature farrowing, an increase in the number of stillborn piglets, and weak neonatal piglets. The PRRS virus is a macrophage-specific virus. CD163 is a molecule found on the surface of macrophages that aids in the establishment of a PRRS infection. Burkard et al. employed CRISPR/Cas9 to remove a tiny segment of the CD163 gene from the pigs' genome, resulting in 32 gene-edited pigs. When exposed to the PRRS virus, these pigs are resistant to infection. The region of CD163 that interacts with the PRRS virus was deleted, but the CD163 proteins' other biological roles were preserved (Burkard *et al.*, 2017). Tuberculosis is a zoonotic disease and a major public health issue that kills roughly 1.5 million people each year (Bhembe, 2016). Bovine tuberculosis is a major concern to

animal husbandry, as there is currently no effective approach for eradicating or controlling the disease. Sp110 nuclear body protein (Sp110) expression increases macrophage death in response to Mycobacterium tuberculosis (Mtb) infection and upregulates host immunity to Mtb, indicating that it could be used to inhibit Mtb growth and transmission. Wu et al. successfully utilized TALEN technology to insert a mouse SP110 gene into the genome of Holstein–Friesian cattle. Transgenic cattle with SP110 gene knock-in showed increased resistance to Mtb infection (Wei *et al.*, 2015).

Improve the quality of livestock products

Cow's milk is nutrient-dense and makes an excellent alternative to breast milk for newborns. However, because newborns' digestive systems are still developing, undigested -lactoglobulin (BLG) in cow's milk might be absorbed and recognized as a pathogen by the infants' immune system, leading in milk allergy. Burkard *et al.* (2017) was the first to use the ZFN approach to create a BLG gene knockout cow to minimize BLG antigenicity and immunogenicity. Co- injected Cas9 mRNA and small guide RNAs (sgRNAs) into goat embryos for the same aim, and successfully developed BLG knock-out goats (2017), which exhibited considerably lower BLG expression in mammary glands than wild-type goats. This research provides a way to increase milk quality while also reducing milk allergies. Foods high in n-3 polyunsaturated fatty acids (n-3PUFAs) can help to lower the risk of cardiovascular and cerebrovascular disease. When expressed within the body of Caenorhabditis worms, the fat-1 gene encodes a fatty acid desaturase that converts n-6 polyunsaturated fatty acids (n-6PUFAs) to n-3PUFAs. As a result, fat-1 animals could potentially be employed to manufacture n-3PUFAs. Li *et al.* (2018) used the CRISPR/Cas9 technology to successfully introduce the fat-1 gene from C. elegans into the porcine Rosa 26 (pRosa26) locus, resulting in fat-1 knock-in pigs. The expression of this gene in pig tissues was validated by gas chromatography analysis, which revealed that fat-1 knock-in pigs had a significantly higher amount of n-3PUFAs and a clearly lower n-6PUFAs/n-3PUFAs ratio. These fat-1 transgenic pigs show potential as a model for studying the therapeutic benefits of n-3PUFAs on illnesses and for increasing the nutritional value of porcine products. Even better, (Zhang *et al.*, 2018) used the CRISPR/Cas9 method to insert the fat-1 gene into the goat MSTN locus, allowing for simultaneous editing of the two genes. The efficiency of simultaneous MSTN knockout and fat-1 knock-in was as high as 25.56 percent, suggesting that the CRISPR/Cas9 system is a feasible gene editing tool in safe animal breeding, according to PCR and sequencing.

Improves animal welfare

Cows' horns are often taken off immediately after birth for the safety of milkers and other animals. Cows' horns are not only uncomfortable to cut with an electric saw or to burn off with a soldering iron, but they are also difficult to manipulate (Kilders and Caputo, 2021). CRISPR/Cas9 has been used to knock-out genes that are responsible for angular growth in cattle. Two hornless Holstein dairy calves have been developed using a combination of in vitro fertilization and embryo transfer techniques to avoid painful horn cutting and increase animal welfare (Croney *et al.*, 2018).

Conclusion

In the modern era, a variety of molecular techniques, in combination with traditional breeding procedures, are regularly having an impact on animal improvement. The development of creative techniques to combat traditional breeding practices, which are slowly becoming obsolete, was aided by the discovery and improvement of genetic markers. Standardized protocols are currently used to establish, develop, and implement a successful procedure, which eventually becomes commonplace among animal breeders around the world. SNPs are beginning to influence and refocus our efforts, and they will undoubtedly play a big role shortly, alongside next-generation sequencing technology. These more recent tools have provided animal geneticists with a powerful tool for incorporating "preferred features" at will while simultaneously eliminating unwanted elements in specific animal populations. The rate of creation of molecular markers is currently incredible, and the tendency predicts that this trend will continue shortly. Molecular markers will, without a doubt, continue to be a useful tool for geneticists and breeders to assess and manipulate genetic potential to generate animals that farmers want and need. These new molecular technologies are unlikely to replace 'traditional' strategies for genetic enhancement. Instead, they will most likely be gradually integrated into current genetic improvement programs that employ effective traditional improvement methods to attain specific goals. To sum up, for molecular genetic tools to contribute positively to long-term livestock production, we need a coordinated strategy that addresses both genetic progress and conservation, as well as overcoming all of the barriers to implementing molecular genetic techniques in routine animal improvement programs. Genome editing is a new genetic engineering tool with the potential to improve quantitative trait responses in livestock breeding programs in the future. ZFNs, TALENs, and CRISPR/Cas9, which were recently developed designed nucleases, allowed for the exact modification of various animal genomes in a straightforward manner, demonstrating the practical application of genome editing techniques. Because no recombinant DNA is incorporated into the animal genome, genome-edited livestock differs from traditional genetically modified animals in that no recombinant DNA is incorporated into the animal genome. This eliminates many of the concerns about the production of genetically modified animals, increasing the likelihood of social acceptance. Genome editing combined with industry standard and reproductive technology will provide a viable method to improving livestock animal.

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