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The Application of CRISPR/Cas9 Technology for Farm Animals: A Review

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ABSTRACT

Livestock animal are important for agriculture economy and biomedical research. They are sources of Milk, meat, carcass, organic manure and other products. The development of genome editing technologies, especially CRISPR-Cas have revolutionized the generation of gene edited farm animals. In this review, we briefly introduce the CRISPR-Cas9 technology and highlight its application on livestock such as human disease modeling, disease resistant animal, and generation of hornless cattle, animal welfare and other agricultural and biomedical related traits which enhance the livestock production in order to meet the increasing demand of food worldwide. The ability to transfer sperm-producing stem cells or spermatogonial stem cells (SSCs) from a donor animal into the testes of a recipient male could have multiple applications. Production of BLG free milk in cattle provides a promising way to those who have allergy to cow milk. The knockdown of myostatin gene in different species like sheep, goat, cattle and pig is very helpful in the economy of meat industry. Besides the several benefits of CRISPR-Cas9 technology, the risk factors and ethics issues related to this technology should be reconsidered before they enter into CRISPR era.

Key words: Cow milk allergy, CRISPR, Disease model, Genome editing, Livestock.

Large animals play important roles in biomedical research. Animal models are crucial for understanding disease pathogenesis and developing novel therapeutic drug and treatments. Livestock animals are important sources of milk, meat, carcass, manure and different value added products. The recently developed genome editing techniques have been used for generation of gene-modified large animals that are used for biological, biomedical and agricultural research and increase the performance of the animals in terms of both qualitative and quantitative. The gene-edited livestock animal has been used as a bioreactor for the production of human biological products such as transgenic pig with human albumin in their blood (Peng *et al*. 2015).

Genome engineering is defined as direct manipulation into the genome that can make specific edition/deletion to the genome by engineered nucleases, which offer a perfect platform to knock out/in and replace the particular DNA fragment, and make accurate genome editing on the genome level. There are three major types of programmed nucleases: zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeat-associated nuclease Cas9 (CRISPR-Cas9). These nucleases produced DNA double-strand breaks (DSBs) at specific sites in the genome by targeted recognition and cleavage. Out of these CRISPR/Cas9 system is the advanced genome editing technology, which are most commonly used nowadays. CRISPR is part of the bacterial genome system, which makes the bacterial cells immune to virus. The CRISPR was first observed in *Escherichia coli* (Ishino *et al*. 1987) and served as an adaptive immune system in bacteria against bacteriophages (Barrangou *et al.* 2007). CRISPR systems, found in 90% of archaeal and 40% of bacterial genomes are highly diverse, with variation in protospacer adjacent motif (PAM) sequences and the number and type of Cas proteins (Grissa *et al*. 2007). The CRISPR system has three types of mechanisms *i.e.* Type I, II and III. In Type I and Type III CRISPR, various types of Cas proteins are participated in the recognition and destruction of target. However, in Type II CRISPR/Cas9 system low number of Cas proteins are involved, so thereby engineering of type II CRISPR system much simpler (Cho *et al.* 2013).

The major question is that why genome editing technology should be used in livestock. Although, conventional methods, such as management of animal health, nutrition and reproduction, make an important contribution to improving the productivity of the animals. However, increasing the demand of food all over growing world, these approaches are not enough to meet the demands, so CRISPR/Cas9 technology emerges as a powerful tool to overcome this problem globally. This main objective of this review article is to explain the basic principle and component of the CRISPR technology and it future application in livestock animal species.

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The CRISPR/cas9 system

The CRISPR/Cas9 system is a part of adaptive immune system of the bacteria. Jinek *et al.* (2012) for the first time showed that this system could be designed as genome editing tool *in vitro* and in 2013 the CRISPR/cas9 genome editing tool demonstrated on the human and mice cells (Cong *et al.* 2013, Mali *el al.* 2013). The CRISPR/Cas9 system contains three main components: Endonuclease (Cas9), CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The Cas9 endonuclease which belongs to Type II CRISPR/Cas9 system, cut the target sites with the help of two RNAs: the crRNA which assign the genomic target for Cas9 and the tracrRNA which serve as a scaffold linking the crRNA to Cas9 and help in processing of maturation of crRNAs into a small single RNA sequence known as the guide RNA (gRNA) or single guide RNA (sgRNA) (Hsu *et al.,* 2014). The specific recognition of target site by sgRNA depends on the protospacer adjacentmotifs (PAM), which is located downstream of 3′ end of the target sequence and after recognition of target site by guide RNA, the cas9 produces a double stand break(DSB) on the complementary locus (Jinek *et al.,* 2012). The generated DSBs will be repaired by two pathways, non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Fig 1). The NHEJ mechanism caused small indels or chromosomal rearrangements and disrupts open reading frames, which leads to knockout of the gene (Jiang and Doudna, 2017). In case of HDR pathway DSB is repaired through sequence homology. Compare to HDR, the NHEJ is more active since HDR mechanism needs a homologous template and is mainly restricted to S and G2 phases of the cell cycle (Xue *et al*. 2015).

The effective endonuclease

For any CRISPR experiment one of the main things is to determine the suitable endonuclease. The spCas9 (*Streptococcus pyogenes*) nuclease is most commonly used but several other type of nucleases such as Cas12a or CasX are also present, which have some unique properties and can be used depending on the objective of the project. There are two domains in the Cas9 nuclease: HNH and RuvC-like domain. The HNH nuclease domain cut the target strand of the nucleotide and RuvC-like domain cut the non-complementary strand (Zhao *et al*. 2019). To increase the specificity and adaptability of the Cas9, researcher reprogrammed the Cas9 into dCas9 nuclease (dead Cas9) (Qi *et al*. 2013). The dCas9 do not have the DNA cleaves activity but can still bind to the target site with the help of guide RNA (gRNA). However the CRISPR/dCas9 system cannot insert or delete the gene, instead it silences the target gene by blocking the transcription process (Moradpour and Abdulah 2020), while CRISPR/Cas9 system produced DSB on the target site. So the CRISPR/dCas9 tool can be used for genetic screening, gene regulation and epigenetic regulation (Adli, 2018). Additionally, the newly RNP based CRISPR tools have also been developed, in which different Cas RNA with sgRNA used for genome editing (Sung *et al.* 2014).

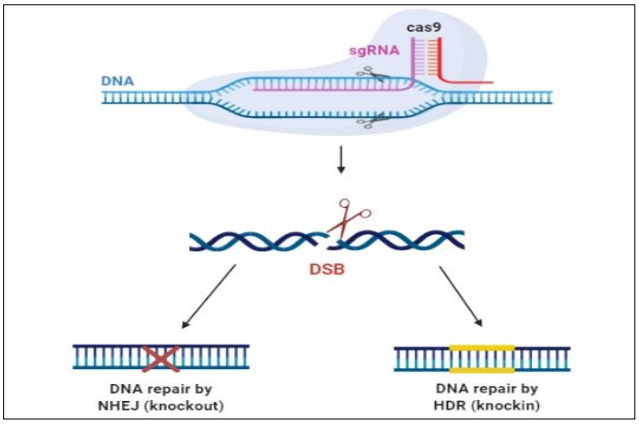

Fig 1: CRISPR-Cas system, consisting of a single guide RNA (sgRNA) designed to direct Cas9 to the desired DNA site and induce double strand breaks (DSB). DNA cleavage results in different gene repair mechanisms as nonhomologous end joining (NHEJ) or homology directed repair (HDR).

The single guide RNA (SgRNA)

The second important component of CRISPR/Cas9 system to edit the genome is selection and design of sgRNA. Generally three rules considered during designing of sgRNA; the sgRNA should have (I) fewest potential off-target matches (II) highest predicted cleavage efficiency (III) Target early exons. These rules increase the probability of frameshift and nonsense mutations that disrupt gene expression (Chekani *et al*. 2020). The target sites for sgRNA are generally 18 bp - 20 bp long and designed immediately to upstream of a Protospacer Adjacent Motif (PAM). The PAM sequence varies depending upon the type of Cas nuclease. The PAM for spCas9 is 5′-NGG-3′ and the PAM for saCas9 is 5′-NNGRRT-3′ (https://info.abmgood.com/crispr). There are many online CRISPR tools and resources are available to design the sgRNA, some of this are list in Table 1 (Chekani *et al*. 2020). These softwares analyze the target sequence and identify all possible 18 bp-20 bp sequences which are immediately after the PAM sequence (5'-NGG) and provide the essential information require for selection of sgRNAs (Thomas *et al*., 2019).

In livestock, the gestation length is long, so after selecting the desired gRNA for target sequence, it is highly recommended to confirm them before producing the edited animals. This analysis can be done in *in vitro* using cultured cells of the same species or directly in embryos (Menchaca *et al*. 2020).

Delivery of CRISPR system

There are mainly three approaches to deliver the CRISPR system *in vitro* or *in vivo*: (I) plasmid encoding both the Cas9 protein and the guide RNA, (II) Cas9 mRNA with guide RNA, and (III) Cas9 protein with guide RNA. The vehicles used for delivery of CRISPR/Cas9 system are divided into three

groups: physical delivery, viral vectors and non-viral vectors. The most common physical delivery methods are microinjection and electroporation. In case of Viral vectors delivery method, specifically engineered adeno-associated virus (AAV) and lenti-virus are used as vehicles (Lino *et al*. 2018). The microinjection delivery method has highest efficiency compare to other CRISPR/Cas9 delivery methods (Yang *et al* 2013). The problem for generation of edited livestock animal is the *in vivo* zygote production (Menchaca *et al*. 2016). *In vitro* embryo production (IVEP) is the preferable method for microinjection delivery of the CRISPR component (Menchaca *et al*. 2020). In microinjection delivery method CRISPR system is directly injected into the cytoplasm of the zygote, thus this method is fast and more efficient and preferable method in large animals (Menchaca *et al*. 2016). The second most preferable method for CRISPR system delivery in livestock is the SCNT (somatic cell nuclear transfer) approach; however the efficiency is low compare to microinjection (Sheets *et al*. 2016).

CRISPR applilcations

Most of the biological and biomedical research is carrying out on rodents but validation and preclinical assessment are performed on large animals. In the last few years, the nuclease-mediated gene editing technologies has been used in livestock breeding; however these approaches are more complicated. The development of CRISPR technology makes the genome editing in large animal easier. Various livestock animal have been produced by using CRISPR technology during the last few years. The major applications of CRISPR technology in farm animals are to increase the productivity like milk, meat and produce disease resistant animal, production of therapeutic proteins into the milk and production of human organs for transplantation (Table 2; Fig 2), which can be describes as below:

Modification of milk composition

β-lactoglobulin (BLG) is a major whey protein of ruminant milk and is considered as allergens when it is devoid of iron in the milk and responsible for cow milk allergy disease (Fiocchi *et al*. 2010). Several methods have been used to diminish the allergenicity of the BLG, including heat treatment, fermentation, hydrolyzed protein desensitization, glycation (Davis *et al* 2001, Ehn *et al*. 2005), however these methods are not so effective. A possible option have been attempted to disrupt the β-lactoglobulin gene expression by targeting BLG gene. Ni *et al*. (2014) have successfully edited the β-lactoglobulin gene by using CRISPR/Cas9 system in goats. Similarly, Zhou *et al*. (2017) also knock out the β-lactoglobulin gene in goat through CRISPR/Cas9 technique and reduced the expression of β-lactoglobulin in milk. The BLG knock out cattle has also been produce by using zinc-fnger nuclease (ZFNs) mRNA (Sun *et al*. 2018) but till now no CRISPR edited BLG knock out cattle has been produced. In a study human lactoferrin (hLF) gene was knocked in at BLG locus through TALEN approach in goat. These edited goats produce the human lactoferrin, a glycoprotein involved in iron adsorption and in non-specific immune reactions in the intestinal tract (Cui *et al*. 2015).

Improvement of meat production and reproduction performance

The well-known example related to meat production is knockout of the myostatin gene (MSTN). This gene secreted a Myostatin protein in muscle tissues, which act as a negatively regulator of muscle growth. The mutation in MSTN

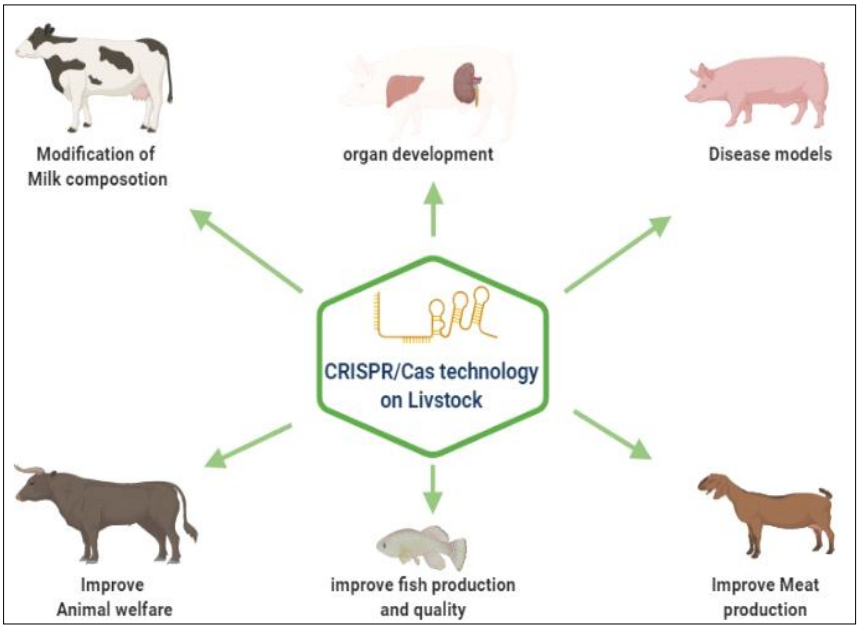

Fig 2: CRISPR/Cas mediated genome editing in major livestock species, enabling improvement of the genetics underlying traits associated with welfare, production, disease resistance and xenotransplantation.

gene leads to double-muscling phenotype in the animals and this was, first reported in cattle and then in sheep, dogs and humans (Zhao *et al*. 2019). The cattle Belgian Blue and Piedmontese or Texel sheep are the well known example of natural mutation in MSTN gene (Bjoern Petersen, 2017). Wang *et al*. (2017) generated myostatin mutation in Erhualian pigs using a combination of CRISPR/Cas9 and somatic cell nuclear and resulted decreased the protein level of myostatin precursor in mutant cloned piglets. Additionally the myostatin knockout pig showed partial double-muscled phenotype such as prominent muscular protrusion, wider back and hip compared with wild-type piglets (Wang *et al*. 2017). So making double-muscling phenotype is an attractive target for genome editing to increase the meat production in livestock.

In 2015 Qian *et al*., MSTN-mutant Meishan pigs through zinc finger nucleases (ZFN) technology that showed an increase in muscle mass by 100% and a decrease in fat deposition compared to wild-type. In 2018 a CRISPR edited MSTN knock out goat has been produce, which showed significantly higher weight gain than that of wild-type (WT) goats (Zhengyi *et al*. 2018). In the same year Zhang *et al*., (2018) knock out the MSTN gene and then knock-in the *fat-1* gene into the goat MSTN locus by using CRISPR technology (Zhang *et al*. 2018). The *fat-1* gene product converts n- 6 PUFA (n- 6 polyunsaturated fatty acid) into n-3 PUFA, so this CRISPR edited goat and pig showed improved muscle growth and also produced nutritious meat by decreasing the ratio of n-6 PUFA to n-3 PUFA, which has been reported as a risk factor for many human diseases (Zhao *et al*. 2019).

The CRISPR technology has also been used for improvement of thermoregulation in livestock. Uncoupling protein 1 (UCP1) play an important role in thermoregulation. The functional UCP1 protein is absent in pig, which makes them liable to cold and prone to fat deposition and results in increase the mortality rate neonates and decreased production efficiency. Zheng *et al*. (2017) knock-in the UCP1 gene in pig through CRISPR technology which showed an improved ability to maintain body temperature during acute cold exposure and also showed reduced fat deposition, which make them valuable resource for the pig industry that can improve pig welfare and reduce economic losses. Park *et al*. (2017) knock out the NANOS2 gene in domestic pigs by using CRISPR/Cas9 technology to generate offspring with monoallelic and biallelic mutations. They found that NANOS2 knockout boar lack a germ line phenotype but other form of testicular development were normal (Park *et al*. 2017). These NANOS2-null boars may provide a good environment to host germ cells from a genetically superior male, and thus broaden his genetic potential (Bjoern Petersen, 2017).

Spermatogonial stem cell transplantation (SSCT) is a technique for transfer of germcells between donor and recipient males that could be used as a tool for biomedical research, preservation of endangered species and transmission of desirable traits in food animal populations. To fully recognize these prospects recipient males must be lacking endogenous germline but have normal testicular structure and somatic cell function competent to allogeneic donor stem cell engraftment and regeneration of spermatogenesis. Ciccarelli *et al*. (2020) show that CRISPR-Cas9 editing of the NANOS2 gene in cattle leads to male germline removal. These findings represent a major advance toward surrogate sires becoming a tool for transmission and regeneration of germplasm in all mammals.

Disease resistance

Mycobacterium bovis which caused tuberculosis in livestock animal especially in cattle is becoming a major risk to the agricultural and public health which causes significant economic loss to the farmers. The NRAMP-1 (natural resistance-associated macrophage Protein-1) gene provided innate resistance to intracellular pathogens such as Mycobacterium, Leishmania, Salmonella and Brucella (Zhao *et al*. 2019). This NRAMP-1 gene has been inserted into the cow genome through CRISPR technology and makes the cow resistant to tuberculosis (Gao *et al*. 2017). Porcine reproductive and respiratory syndrome (PRRS) is the most economically important disease of swine all over

Table 1: List of CRISPR guide RNA designing tool (Chekani *et al*. 2020).

Name	URL
Addgene	http://www.addgene.org/crispr/
Benchling	https://benchling.com/crispr
Breaking Cas	http://bioinfogp.cnb.csic.es/tools/breakingcas/
Broad Institute GPP	https://portals.broadinstitute.org/gpp/public/
CHOPCHOP	http://chopchop.cbu.uib.no/
CRISPOR	http://crispor.net/
Cris Flash	https://github.com/crisflash/crisflash
Deskgen	https://www.deskgen.com
E-CRISP	http://www.e-crisp.org/E-CRISP/
Microsoft research cRISPR	https://crispr.ml
RGEN tools	http://www.rgenome.net/
Synthego	http://design.synthego.com
WTSI genome editing (WGE)	https://www.sanger.ac.uk/htgt/wge/

the world, which is cause by porcine reproductive and respiratory syndrome virus (PRRSV). The vaccines have been developed for this disease but unable to control the disease. The CD163 is the receptor for entry of PRRSV into cells. So in pig the CD163 protein encoding gene has been knock out through CRISPR technique, results in no clinical signs of PRRS were observed in edited pigs which demonstrated that a single gene deletion creates PRRSV resistant pigs (Whitworth *et al*. 2016). Niemann-PickC1-Like-1 (NPC1L1) protein is potent cholesterol absorption inhibitor that lowers blood cholesterol in humans and highly expressed in human liver. The NPC1L1 gene has been introduced into the pig genome to produce animal for understanding of cardiovascular and metabolic diseases (Wang *et al*. 2015).

Biomedical application

The most crucial issue for the patients awaiting organ for transplantation is the availability and rejection of the allograft. Xenotransplantation is one of the solution in which the transplantation of animal cells, tissues or organs could replace an injured tissue or whole organ in humans. Pigs are the best choice for human organ development because anatomical and physiological similarities to the human organs and are cost-effective in breeding (Zhao *et al*. 2019) but the major problems are the immune rejection and potential cross species infection. So to overcome these problems scientists have been introduce human organ development gene into the pig genome to produce human organs into the pig. Wu *et al*. (2017) successfully inactivated the pancreatogenesis in pig embryos *via* zygotic co-delivery of Cas9 mRNA and dual sgRNAs targeting the PDX1 gene, which makes the pigs suitable for the xeno-generation of human tissues and organs.

Another problem related to Xenotransplantation is the risk of retroviral transmission from pig to human because Porcine endogenous retroviruses (PERVs) are presented as integrated form in the genome of all pigs, so they remains in all tissues and organs and can be inherit. Yang *et al*. (2015) successfully eradicated all PERVs in a porcine kidney epithelial cell line (PK15) and demonstrated a >1000-fold reduction in PERV transmission to human cells, by using CRISPR/Cas9. Similarly with the help of CRISPR/cas9 technique, Niu *et al*. (2017) inactivated all of the PERVs in a porcine primary cell line and produced PERV-inactivated pigs via somatic cell nuclear transfer and addressed the safety concern in clinical xenotransplantation.

Generating B cell deficient mutant is the first step to produce human antibody repertoires in large animal models. IgM heavy chain gene is crucial for B cell development and differentiation. This IgM heavy chain gene has been knock-out in pig through CRISPR technology, resulted in a B cell-deficient pig (Chen *et al.* 2015). This study highlights the potential of CRISPR technology in pig, which can used as animal model for study of human diseases. Human serum albumin (HSA) is the most abundant plasma protein that plays important role in homeostatic functions in human. Several approaches have been used to produce recombinant human serum albumin (rHSA), however not so effective because of separation and purification of the rHSA are problematic. To overcome these problems Peng *et al*. (2015) knocking human albumin cDNA into swine albumin locus to produce rHSA in pigs, which can used as bioreactor for the production of human albumin.

The neuronal ceroid lipofuscinoses (NCLs/CLN1) are a group of inherited, neurodegenerative, lysosomal storage disorders that affect children and young adults. The more critical infantile forms of this disease are due to mutations in the palmitoylprotein thioesterase 1 (PPT1) gene, which reduces the child's lifespan to approximately 9 years of age. Eaton *et al.* (2019) produced disease-causing PPT1 (R151X) human mutation into the orthologous sheep locus through CRISPR/Cas9 technology and generated a sheep model for the study of CLN1 disease.

Table 2: List of CRISPR/Cas9 edited livestock animals for the different purposes (Zhao *et al.* 2019; Bjoern Petersen, 2017).

Species	Gene	Modifications	Applications
Pig	Npc1l1	KO	Disease model for cardiovascular and metabolic diseases
Pig	ApoE/LDLR	KO	Disease model for cardiovascular diseases
Pig	vWF	KO	Disease model for vWD
Pig	TP53/PTEN/APC	KO	Disease model for lung cancer
Pig	TPH2	KO	Disease model for 5-HT deficiencyinduced behavior abnormality
Pig	Huntingtin	KI	Disease model for HD
Dog	ApoE	KO	Disease model for cardiovascular disease
Dog	MSTN	KO	Improve muscle growth, new strains
Pig	GGTA1/CMAH	KO	Xenotransplantation
Pig	PERV	KO	Xenotransplantation
Goat	MSTN	KO	Goat meat production, composition and quality
Goat	MSTN (fat-1)	KI	Goat meat production, composition and quality
Pig	UCP1 (mouse UCP1)	KI	Pig meat production, composition and quality
Pig	CD163	KO	Disease resistance to PRRSV
Cattle	NRAMP1	KI	Disease resistance to tuberculosis

KO- Knock out; KI- Knock in.

Application in aquaculture

The fish farming contributes billions of dollars to the world economy. The genome editing technology has also been demonstrated in area. Escaping of farmed salmon fish into wild stocks is a major threat to the genetic integrity of wild populations. To overcome this problem dnd-gene (required for germ cell survival in vertebrates) has been knock-out by using CRISPR-Cas9 system (Wargelius *et al*. 2016). Similarly genome edited ridgetail white prawn has been produced by microinjection of Cas9 mRNA (Zhang *et al*. 2018). Gratacap *et al*. (2020) successfully edited two target genes with high efficiency in an EGFP-Cas9 stable CHSE cell line, specifically the exogenous, integrated EGFP and the endogenous RIG locus, which were successfully demonstrated for antibiotic selection.

The fish oil especially eicosapentaenoic acid and docosahexaenoic acid are very useful to the prevention of inflammatory and cardiovascular diseases in humans. Atlantic salmon can synthesize these polyunsaturated fatty acids (PUFAs) to a limited degree and unable to meet the increasing demands for omega-3 PUFAs. Datsomor *et al.* (2019) partially knockout the elovl2 gene in Atlantic salmon through CRISPR/Cas9 technology and showed reduced levels of docosahexaenoic acid (22:6n-3) and accumulation of docosapentaenoic acid (22:5n-3) in the liver, brain and white muscle and suggested that sterol regulatory element binding protein-1 (*srebp-1*) as a main regulator of endogenous PUFA synthesis in Atlantic salmon. The myostatin (MSTN) gene edited catfish (*Ictalurus punctatus*) has been generated through CRISPR/Cas9 approach and showed more muscle cells in fry head than controls and the mean body weight of gene-edited fry increased by 29.7%. These results clearly showed that CRISPR/Cas9 is a highly efficient tool for editing the catfish genome to promote catfish genetic improvement and productivity (Khalil *et al.* 2017).

From these data it is clearly indicated that CRISPR/Cas9 provided new solutions and opportunities in this area.

Application in animal welfare

The horned animal in the farm caused many problem such as they injure the animal, require more space for feeding, are dangerous to handle and transport than hornless animals. To overcome these problem farmers generally destruct the horn-producing cells before they grow and attach to the skull (disbudding) to prevent horn growth. But this practice is very painful to the animal. In 2016 Carlson *et al*., has been produced the hornless dairy cattle by transcription activator-like effector nucleases (TALENs) approach (Carlson *et al*. 2016) but no such type of animal has been produce through CRISPR technology which is more efficient and easy technique compare to other genome editing technology.

In livestock industries, selection of sex is usually depends on the type of production *e.g.* milk from cows and meat from bulls. In pigs, 5-100% of boars develop an unpleasant meat taint, which is caused by the two main factors: one is the androstenone, synthesized in the testis and second is the skatole produced by microbial degradation of the amino acid L-trypothopan in the colon. The simple method to remove this odor is to castrate piglets shortly after birth and this is generally carried out by surgically without anesthesia. So, alternatives approaches to surgical castration are required to address animal welfare concerns. In this regards CRISPR/Cas9 technique provides a great opportunities to pre-determination of sex in pigs (Kurtz *et al*. 2019).

CONCLUSION

Genome editing by CRISPR/Cas9 system has become a prevailing tool in biological research. It have the ability to generate site-specific indels to the genome, which allows help in the identification of gene function, allelic variants between breeds or species, or to create novel phenotypes. Compare to other genome editing techniques (ZFN and TALEN), CRISPR/Cas9 system is more efficient and easy to handle. The CRISPR technology has been successfully used for generation of many valuable animals for human disease models, xeno-transplantation and the agricultural economy such as improves the milk composition, meat production and disease resistant livestock animals. Mostly this tool used on the pig because of anatomical and physiological similarities to human, however very less effort has been made on cattle. Buffalo contribute more than 50% milk production in India and like cow milk; buffalo milk also contains beta-lactoglobulin which act as allergen in many children and adults. So to overcome this problem, CRISPR technology could be used for the production of beta-lactoglobulin knock out buffalo in the future.

REFERENCES

Adli, M. (2018). The CRISPR tool kit for genome editing and beyond. Nat Commun. 9(1): 1911.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science. 3: 1709-1712.

Bjoern Petersen (2017). Basics of genome editing technology and its application in livestock species. Reprod Domest Anim. 52(3): 4-13.

Carlson, D.F., Lancto, C.A., Zang, B., *et al.* (2016). Production of hornless dairy cattle from genome-edited cell lines. Nature Biotechnology. 34(5): 479-481.

Chekani, A.S., Mombeni, E.G., Birhan, M. and Yousefi, M. (2020). CRISPR/Cas9 gene editing technology and its application to the coronavirus disease (COVID-19): A review. Journal of World's Poultry Research. 10: 1-09.

Chen, F., Wang, Y., Yuan, Y., *et al.* (2015). Generation of B cell-deficient pigs by highly efficient CRISPR/Cas9-mediated gene targeting. Journal of Genetics and Genomics. 42(8): 437-444.

Cho, S., Kim, S., Kim, J. *et al.* (2013). Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31: 230-232.

Ciccarelli, M., Giassetti, M.I., Miao, D., Oatley, M.J., Robbins, C., Lopez-Biladeau, B., Waqas, M.S., Tibary, A., Whitelaw, B., Lillico, S., Park, C.H., Park, K.E., Telugu, B., Fan, Z., Liu, Y., Regouski, M., Polejaeva, I.A., Oatley, J,M. (2020). Donor-derived spermatogenesis following stem cell transplantation in sterile NANOS2 knockout males. Proc. Natl. Acad. Sci. USA. 117(39): 24195-24204.

Cong, L., Ran, F.A., Cox, D., *et al.* (2013). Multiplex genome engineering using CRISPR/Cas systems. Science. 339(6121): 819-823.

Cui, C., Song, Y., Liu, J. *et al.* (2015). Gene targeting by TALEN-induced homologous recombination in goats directs production of β-lactoglobulin-free, high-human lactoferrin milk. Sci. Rep. 5: 10482.

Datsomor, A.K., Zic, N., Li, K. *et al.* (2019). CRISPR/Cas9-mediated ablation of elovl2 in Atlantic salmon (*Salmo salar* L.) inhibits elongation of polyunsaturated fatty acids and induces Srebp-1 and target genes. Sci. Rep. 9: 7533.

Davis, P.J., Smales, C.M., James, D.C. (2001). How can thermal processing modify the antigenicity of proteins? Allergy. 56: Suppl. 67: 56-60.

Eaton, S.L., Proudfoot, C., Lillico, S.G. *et al.* (2019). CRISPR/Cas9 mediated generation of an ovine model for infantile neuronal ceroid lipofuscinosis (CLN1 disease). Sci Rep. 9: 9891.

Ehn, B.M., Toomas, A., Esbjo R.T., Ulf, B. and Bo, E. (2005). Modification if Ige binding to β-Lactoglobulin by fermentation and proteolysis of cow's milk. Journal of Agricultural and Food Chemistry. 53(9): 3743-3748.

Fiocchi, A., Brozek, J., Schünemann, H., Bahna, S.L., von Berg, A., Beyer, K., Bozzola, M., *et al.* (2010). World Allergy Organization (WAO) Special Committee on Food Allergy. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. Pediatr Allergy Immunol. 21 Suppl. 21: 1-125.

Gao, Y., Wu, H., Wang, Y. *et al.* (2017). Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. Genome Biol. 18: 13.

Gratacap, R.L., Regan, T., Dehler, C.E. *et al.* (2020). Efficient CRISPR /Cas9 genome editing in a salmonid fish cell line using a lentivirus delivery system. BMC Biotechnol. 20: 35.

Grissa, I., Vergnaud, G. and Pourcel, C. (2007) The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics. 8: 172 (2007).

Hsu, P.D., Lander, E.S., Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell. 157(6): 1262-1278. https://info.abmgood.com/crispr-crash-course-week-2?utm_campaign=CRISPR. Design and Validation.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., Nakata, A., (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli* and identification of the gene product. Journal of Bacteriology. 169(12): 5429-5433.

Jiang, F. and Doudna, J.A. (2017). CRISPR–Cas9 Structures and Mechanisms. Annual Review of Biophysics. 46(1): 505-529.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 337(6096): 816-821.

Khalil, K., Elayat, M., Khalifa, E. *et al.* (2017). Generation of myostatin gene-rdited channel catfish (*Lctalurus punctatus*) *via* Zygote Injection of CRISPR/Cas9 System. Sci. Rep. 7: 7301.

Kurtz, S. and Petersen, B. (2019). Pre-determination of sex in pigs by application of CRISPR/Cas system for genome editing. Theriogenology. 137. 10.1016/j. Theriogenology. 05.039.

Lino, C.A., Harper, J.C., Carney, J.P., Timlin, J.A. (2018). Delivering CRISPR: A review of the challenges and approaches. Drug Deliv. 25(1): 1234-1257.

Mali, P., Luhan, Y., Esvelt, K.M., Aach, J., Guell, M., Dicarlo, J.E., Norville, J.E., Church, G.M. (2013). RNA-guided human genome engineering *via* Cas9. Science. 15: 339(6121): 823-826.

Menchaca, A., Anegon, I., Whitelaw, C.B.A., Baldassarre, H., Crispo, M. (2016). New insights and current tools for genetically engineered (GE) sheep and goats. Theriogenology. 86(1): 160-169.

Menchaca, A., dos Santos Neto, P.C., Mulet, A.P., Crispo, M. (2020). CRISPR in livestock: From editing to printing. Theriogenology. 01.06: 3.

Moradpour, M. and Abdulah, S.N.A. (2020). CRISPR/dCas9 platforms in plants: Strategies and applications beyond genome editing. Plant Biotechnology Journal. 18: 32-44.

Ni, W., Qiao, J., Hu, S., *et al.* (2014). Efficient gene knockout in goats using CRISPR/Cas9 system. PLoS One. 9(9): e106718.

Niu, D., Wei H.J., Lin, L., George, H., Wang, T., Lee, I.H., *et al.* (2017). Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science. 357: 1303-7.

Park, K., Kaucher, A., Powell, A. *et al.* (2017). Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. Sci. Rep 7: 40176.

Peng, J., Wang, Y., Jiang, J. *et al.* (2015). Production of human albumin in pigs through CRISPR/Cas9-mediated knockin of human cDNA into swine albumin locus in the Zygotes. Sci. Rep. 5: 16705.

Qi, L.S., Larson, M.H., Gilbert, L.A., *et al.* (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 152(5): 1173-1183.

Qian, L., Tang, M., Yang, J. *et al.* (2015). Targeted mutations in myostatin by zinc-finger nucleases result in double-muscled phenotype in Meishan pigs. Sci. Rep. 5: 14435.

Sheets, T.P., Park, C.H., Park, K.E., Powell, A., Donovan, D.M., Telugu, B.P. (2016). Somatic cell nuclear transfer followed by CRIPSR/Cas9 microinjection results in highly efficient genome editing in cloned pigs. Int. J .Mol. Sci. 17(12): 2031.

Sun, Z., Wang, M., Han, S. *et al.* (2018). Production of hypoallergenic milk from DNA-free beta-lactoglobulin (BLG) gene knockout cow using zinc-finger nucleases mRNA. Sci. Rep. 8: 15430.

Sung, Y.H., Kim, J.M., Kim, H.T., *et al.* (2014). Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. Genome Res. 24(1): 125-131.

Thomas, M., Smith, D.P. and Iyer, V. (2019). Best practice for CRISPR design using current tools and resources. Methods. 15: 164-165: 3-17.

Wang, K., Tang, X., Xie, Z., Zou, X., Li, M., Yuan, H., *et al.* (2017). CRISPR/Cas9-mediated knockout of myostatin in Chinese indigenous Erhualian pigs. Transgenic Res. 26: 799-805.

Wang, Y., Du, Y., Shen, B. *et al*. (2015). Efficient generation of gene-modified pigs via injection of zygote with Cas9 sgRNA. Sci. Rep. 5: 8256.

Wargelius, A., Leininger, S., Skaftnesmo, K.O., *et al*. (2016). *Dnd* knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. Sci. Rep. 6: 21284.

Whitworth, K., Rowland, R., Ewen, C. *et al*. (2016). Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotechnol. 34: 20-22.

Wu, J., Vilarino, M., Suzuki, K. *et al*. (2017). CRISPR-Cas9 mediated one-step disabling of pancreatogenesis in pigs. Sci. Rep. 7: 10487.

Xue, H.Y., Ji, L.J., Gao, A.M., Liu, P., He, J.D., Lu, X.J. (2015). CRISPR-Cas9 for medical genetic screens: Applications and future perspectives. J. Med Genet. 53(2): 91-7.

Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L., Jaenisch, R. (2013). One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell. 154(6): 1370 1379.

Yang, L., Güell, M., Niu, D., George, H., Lesha, E., Grishin, D., *et al*. (2015). Genomewide inactivation of porcine endogenous retroviruses (PERVs). Science. 350: 1101-4.

Zhang, J., Song, F., Sun, Y., Yu, K., Xiang, J. (2018). CRISPR/Cas9-mediated deletion of EcMIH shortens metamorphosis time from mysis larva to postlarva of Exopalaemon carinicauda. Fish Shellfish Immunol. 77: 244-251.

Zhang, J., Meng-Lan, C., Yong-Wei, N., Dai, B., Fei-Ran, L., Dong-Jun, L., Hao, L., Ming, C. (2018). CRISPR/Cas9-mediated specific integration of *fat-1* at the goat MSTN locus. FEBS J. 285(15): 2828-2839.

Zhao, J., Lai, L., Ji, W., Zhou, Q. (2019). Genome editing in large animals: current status and future prospects. National Science Review. 6(3): 402-420.

Zheng, Q., Lin, J., Huang, J., Zhang, H., Zhang, R., Zhang, X. *et al*. (2017). Reconstitution of UCP1 using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and improves thermogenic capacity. PNAS. 114(45): 9474-9482.

Zhengyi, H., Zhang, T., Jiang, L., Zhou, M., Wu, D., Mei, J. and Cheng, Y. (2018). Use of CRISPR/Cas9 technology efficiently targetted goat myostatin through zygotes micro injection resulting in double-muscled phenotype in goats. Biosci. Rep. 38(6). BSR20180742.

Zhou, W, Wan, Y, Guo, R., *et al*. (2017). Generation of beta-lactoglobulin knock-out goats using CRISPR/Cas9. PLoS One. 12(10): e0186056.