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## Smart bomb: Potential applications and future perspectives in aquaculture

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### Abstract

Antibiotics have acted as a boon for treatment of infectious diseases. Nowadays, antibiotic usage is restricted due to the development of antibiotic resistance and indiscriminate extermination of both beneficial and pathogenic bacteria. Antibiotics pose a threat to the digestive system leading to weakened immunity by eradication of beneficiary bacteria. The emergence and increasing prevalence of multidrug-resistant bacteria pathogens in aquatic system emphasizes the need of innovative antimicrobial strategies to combat the currently shrinking therapeutic options against the antibiotic-resistant bacteria. Microbiologists have thus developed a 'smart bomb' technique that can serve as a best alternative to the menace by identifying and eliminating the specific pathogenic strains by exploiting the novel strategy of CRISPR-Cas system. This targeted approach is gaining new insights inviting extensive research and practical implications in allied sectors. This article suggests the potentiality of this nascent technique in conferring a promising and propitious contribution to the aquaculture domain against the perilous fish bacterial diseases.

**Keywords:** Antibiotic-resistance, bacteria, *smart bomb*, CRISPR-Cas System, aquaculture

### 1. Introduction

Antibiotics have been considered as life savers but its misuse has led to emergence and prevalence of the antibiotic resistant bacteria population [1]. With antibiotic usage, both the harmful and beneficial bacteria are indiscriminately destroyed leading to antibiotic resistance, mineral loss, diarrhea, parasitic infection, development of allergies, lactose intolerance, cancer, depression, inflammation of the gut, chronic fatigue syndrome, leaky gut, yeast over growth and a weakened immune system [2]. After the antibiotic treatment has concluded, the surviving resistant bacteria in the body begin to multiply creating its own population imparting multiple drug resistance with a possibility of returning of these genes to the human population through fish consumption [3]. The indiscriminate killing of both beneficial and pathogenic bacteria by usage of antibiotics in aquatic system causes a major threat highlighting the need of triggering a targeted killing approach. Therapies capable of selective elimination may help to re-establish the normal flora and provide long-term protection [4]. A new antibiotic "smart bomb" that can target specific strains of bacteria could provide the next generation antibiotic drugs needed to stave off the threat of antibiotic resistant bacteria reflecting tremendous potential in resisting infections caused by multi-drug resistant bacteria [5]. This article discusses about the CRISPR-Cas technique involved in the production of *smart bomb*, the information regarding its use in allied sectors and its potential applications that can be explored in the field of aquaculture for combating the threat of antibiotic resistance by targeting only the pathogenic bacteria and leaving the beneficial microbiota untouched.

### 2. Why does the beneficial bacterial population need to be safe?

The beneficial roles of bacteria are listed as follows:

- Bioremediation where microbial activity can be exploited for degradation/removal of environmental pollutants from the environment.
- Bioaugmentation that enhances populations of microorganisms capable of pollutant removal either by adding microorganisms that naturally contain catabolic genes or those that have been genetically modified (GMOs).

- Immunomodulatory role in which bacterial extracts (specific and non-specific stimulating agents) indicated in the prevention, treatment of respiratory infections and adjuvants in case of infections resistant to common antibiotics or in the treatment of complications [6]. OM 85 is an immuno-stimulant extracted from 8 bacterial pathogens (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus viridans* and *Neisseria catarrhalis*) that significantly reduced the rate of upper respiratory tract infections (URTIs), particularly in children [7].
- The microbiota allows the induction of protective responses to pathogens and the maintenance of regulatory pathways involved in the maintenance of tolerance to innocuous antigens [8] of the host immune system.

### 3. Smart Bomb: Target definitive therapy to known pathogen

Conventional antibiotic treatments eliminate indiscriminately beneficial bacteria that are essential to digest food and produce healthy vitamins along with pathogenic bacteria perturbing the microbial harmony leading to unintended consequences encouraging susceptibility to various opportunistic infection and diseases [9]. CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) systems in bacteria and archaea employ CRISPR RNAs to specifically recognize the complementary DNA of foreign invaders, leading to sequence specific cleavage or degradation of the target DNA [5] that also apparently bypasses the antibiotic resistance mechanism and offers tremendous potential in the realms of genome editing, gene regulation and antimicrobials [10].

#### 3.1 How does the “Smart bomb” work?

A distinct mechanism of horizontal transfer of genetic elements has been elucidated in prokaryotes that protect microbial cells against exogenous nucleic acids of phage or plasmid origin [11]. Bacteriophages, a large group of virus, known to infect bacteria transfers its DNA to the bacterial cell, hijacks the bacterial DNA-replicating machinery to make multiple copies of itself, and then escapes after causing lysis of the host cell [12]. About 40% of sequenced bacterial genomes contain a set of CRISPR/Cas genes, an immune mechanism to protect themselves against invading phages. The “Smart Bomb” takes advantage of a part of an immune system present in many bacteria called the CRISPR-CAS system [13].

#### 3.2 Current status of smart bomb and CRISPR/Cas technology in allied sectors:

- **Smart Bomb in Cancer** - T-DM1 is the first “smart bomb” designed to treat breast cancer that target specific cell types, such as cancer cells, without causing significant damage to other surrounding cells, tissues and organs. In order to target colchicines directly to tumors, the researchers enlisted the use of powerful enzymes ‘matrix metalloproteinase (MMP)’ that can dissolve the extracellular matrix used as a substrate for growth by the cancer cells (during a process called angiogenesis), providing essential oxygen and nutrients [14]. To exploit this, the researchers attached long molecular “tails” to individual colchicine molecules. These modified colchicines (called ICT2588) was less toxic to healthy

cells but if the “tail” was hacked off by MMP enzymes then it was activated into a potent cell-killing drug [11].

Trastuzumabemtansine (T-DM1) is an antibody-drug conjugate comprising trastuzumab (Herceptin) linked to the chemotherapy agent emtansine (DM1) that specifically targets HER2-positive cancers, not only inhibiting HER2 signaling but also releasing emtansine directly only into the tumor cells thus sparing normal healthy cells the drug’s cytotoxic effects (a so-called ‘smart bomb’ approach) [15, 16].

- **A nanoscale Smart Bomb to target childhood leukemia-** Here, the target of the smart bomb is a defective gene that results in production of a dysfunctional CD22 due to deletion of exon 12 (CD22ΔE12) which causes cancer stem cells to proliferate and resist chemotherapy in B-lineage acute lymphoblastic leukemia (ALL) without affecting the surrounding tissue [17]. A unique polypeptide-based nanoparticle formulation of CD22ΔE12-siRNA as an RNAi therapeutic candidate targeting CD22ΔE12 that is capable of delivering its siRNA cargo into the cytoplasm of leukemia cells causing effective CD22ΔE12 depletion and marked inhibition of leukemic cell growth. Further development and optimization of this nanoparticle may facilitate the development of an effective therapeutic RNAi strategy against a paradigm shift in therapy of aggressive or chemotherapy-resistant B-lineage lymphoid malignancies [18].
- **Smart Bomb in Mouth wash** - Current mouthwash products act like nuclear bombs killing good and bad bacteria indiscriminately and can effectively eliminate dental cavities [11]. It targets and eliminates only *S. mutans* bacteria that are the main cause of tooth decay while leaving the good bacteria that helps fight tooth decay unhampered. The scientists used new antimicrobial technology known as specifically targeted anti-microbial peptides [19, 20].
- **Smart bomb in detecting brain tumor-** An experimental drug (MP-MUS) attacks brain tumor tissue by crippling the cells’ energy source, mitochondria by infiltrating both inner and outer membranes after being converted from an inactive, non-toxic form to an active toxic form by the enzyme MAO-B (over-expressed in brain tumor cells) [21]. Once inside, the drug damages mitochondrial DNA which cannot be repaired and has passed early tests in animal models and human tissue cultures. Importantly, MP-MUS were found to be selectively toxic towards glioma cells. In the concentration range of 150-180 nm, MP-MUS killed 90-95% of glioma cells but stimulated the growth of normal human astrocytes [22].
- **Combating Viral Diseases-** The Cas9-based DNA editing system is being exploited to help combat viral diseases through the identification of human genes linked to viral replication and the direct targeting of DNA viruses within the human body [23] and mounting intracellular defense against HIV-1 infection [24]. The engineered cells expressing HIV-targeted CRISPR/Cas9 can disrupt viral DNA integrated into the host genome and simultaneously prevent new viral infection.
- **Disease Correction-** CRISPR-Cas has the ability to engineer genomic DNA in cells and organisms in the investigation of disease processes driving therapeutic innovations [25] and correct disease-causing genetic mutations [26]. More recently, a rare, but fatal, genetic

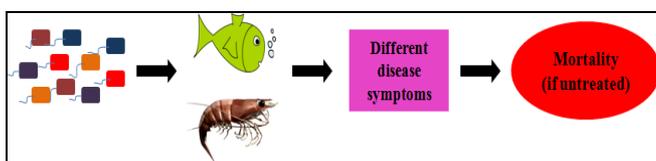
condition caused by a mutation of the fumaryl acetoacetate hydrolase (FAH) gene in liver cells was corrected using CRISPR-Cas9 [27].

- **Study of human diseases-** It has been demonstrated that CRISPR/Cas9- mediated genome editing allows rapid, efficient and simultaneous knockout of several genes in mouse embryonic stem cells [28, 29]. This can help in studying in vivo gene functions underlying different disease mechanisms and biological processes. Recent studies has shown that by using Adeno-associated Virus 9 to deliver single-guide RNA (sgRNA) against the *Myh6* gene (responsible for cardiomyopathy and heart failure), genomic insertions and deletions can be rapidly induced in transgenic mouse models [30].

#### 4. Antibiotic resistant bacteria in Aquaculture sector

Disease outbreaks caused by bacterial diseases are considered to be a key constraint in the aquaculture sector resulting in highly unpredictable survival rates and significant stock losses. The bacterial diseases symptoms in fish include inactivity, loss of color, frayed fins, bloated body, cloudy eyes, open sores, abscesses, red streaks throughout the body, reddening or inflammation of the skin, fins or internal organs, bulging eyes and difficulty in breathing eventually leading to mortality and consequential loss [31]. Antimicrobials are administered into the aquatic system by the application of medicated feed, adding antibiotics into feed as growth promoters, for therapeutic and prophylactic purposes [32, 33] and release to the water during treatment of fish bacterial diseases. The frequent use of one or more antibiotics has led to development of antibiotic resistance in bacteria which accompanies unresponsiveness and ineffectiveness to multiple drugs. Transfer of these antibiotic resistant bacteria via the food chain is a significant health issue and of major concern. Multiple drug resistance has been reported in a number of studies of fish pathogens and aquaculture environments [34, 35, 36], shrimp ponds [37], shellfish [38] and aquaculture environments [34, 37, 39, 40]. Subsequently, fish contamination with antibiotic-resistant bacteria can be a major threat to public health as it can be transferred to other bacteria of human clinical significance [41]. Plasmids that carry multidrug-resistant determinants have been shown to be transferable to *E. coli* from *A. salmonicida*, *A. hydrophila*, *E. tarda*, *Citrobacter freundii*, *P. damsela* spp. *piscicida*, *V. anguillarum* and *V. salmonicida* [42, 43] via horizontal gene transfer.

Antibiotic resistance has been reported in *A. salmonicida*, *A. hydrophila*, *E. tarda*, *Citrobacter freundii*, *Yersinia ruckeri*, *P. damsela* ssp *piscicida*, *V. anguillarum*, *V. salmonicida*, *F. psychrophilum* and *P. fluorescens* [43]. Antibiotic resistance would inexplicably result in accumulation of resistant traits in bacteria convincing antibiotic treatment failures in humans or prolonged duration of illness, increased frequency of bloodstream infection, increased hospitalization or increased mortality [44].



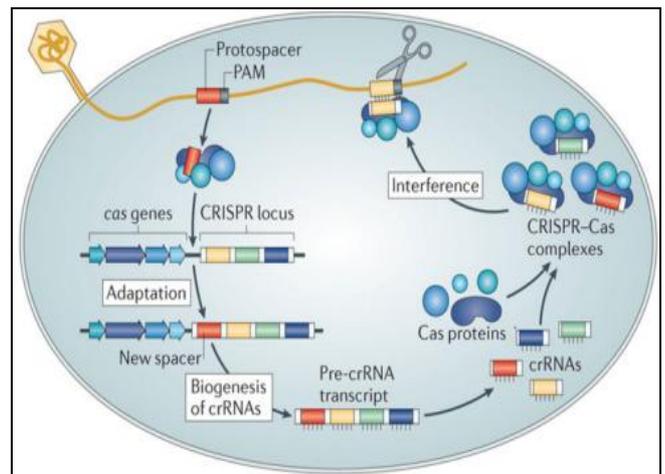
**Fig 1:** Fate of bacterial disease inflicted aquatic organisms

#### 5. CRISPR-Cas systems for alleviating use of antibiotics in aquaculture

Genome targeting can be employed for the sequence-specific and titratable removal of individual bacterial strains and species that consists of two main components: (i) the CRISPR locus and (ii) Cas genes, encoding CRISPR-associated (cas) proteins [11, 45].

This system can function in three specific ways:

- Adaptation or immunization (involving the acquisition of spacers)- This process involves the separation of arrays of short repeats of the CRISPR locus by highly variable non-repetitive DNA sequences called “spacers” derived from exogenous DNA targets known as protospacers constituting the CRISPR RNA (crRNA) array.
- Biogenesis of CRISPR RNA (crRNA) encoded by the repeat-spacer regions-The CRISPR locus is transcribed as a long primary pre-crRNA transcript and precisely processed to produce an assembly of small mature crRNAs and;
- Target interference-crRNAs act as guide RNAs for different interference modules that target and cleave DNA/RNA after annealing to the complementary protospacer sequence within nucleic acid of the invading element [20, 46, 47, 48]. crRNA guide a set of multifunctional protein complex (Cas proteins) to complementary invading nucleic acid resulting in target interference [49] by cleavage in a sequence specific manner to ensure cell defense [50].



**Fig 2:** The Mechanism of CRISPR-Cas system [51]

Varying in their specific target and mechanism of action, three types of CRISPR-Cas systems have been defined: Type I systems cleave and degrade DNA through the action of a 3'-5' exonuclease activity, type II systems cleave DNA, and type III systems cleave DNA or RNA [52]. Using the *E. coli* type I system as a model led to potent removal of *E. coli* cells as long as the target sequence contained a Protospacer -Adjacent Motif (PAM) and was complementary to the spacer regardless of the genomic location, strand, or transcriptional activity of the target sequence [5]. Researchers have already tested the antibiotic on *Salmonella* cultures and are currently working on developing an effective method to administer an antibiotic in a clinical setting [53]. The specificity of targeting with CRISPR RNAs could be achieved even between different strains that shared 99 percent DNA homology whether in pure or mixed cultures using genomic sequence information [5].

### 5.1 CRISPR/Cas9 – A nascent Genome Editing revolution

Cas9-based tools constitutes a next-generation genome editing method and is particularly grabbing attention due to its inexpensive, simplicity, accessibility and high-throughput functional genomics [54], interrogation of gene function of mammalian cells [55], performing genome-wide screens, creating disease models and perhaps developing therapeutic agents [56]. The core RNA-guided Cas9 endonuclease in the type II CRISPR system has been harnessed to realize gene mutation and DNA deletion and insertion, as well as transcriptional activation and repression, with multiplex targeting ability, just by customizing 20-nucleotide RNA components [57].

CRISPR-Cas9 technology can be used to introduce random or targeted mutations into any portion of the genome such as the coding region, promoter, or enhancer regions of genes [58].

Gene knock out technologies with minimal off-target effects and knocking out individual gene expression at the genomic level is mediated by CRISPR-Cas9 through the use of truncated guide RNAs (truncated within the crRNA-derived sequence), by adding two extra guanine (G) nucleotides to the 5' end [59, 60] or with the use of 'paired nickases' [61].

In endogenous CRISPR/Cas9 system, mature crRNA is combined with transactivating crRNA (tracrRNA) to form a tracrRNA: crRNA complex that guides Cas9 to cleave DNA and generate double strand breaks at target sites. At the target site, CRISPR/ Cas9-mediated sequence-specific cleavage requires a DNA sequence protospacer matching crRNA and a short protospacer adjacent motif (PAM) [62]. This newly emerging tool not only promises to revolutionize the field of genetics, but also has direct application to the treatment of disease [63].

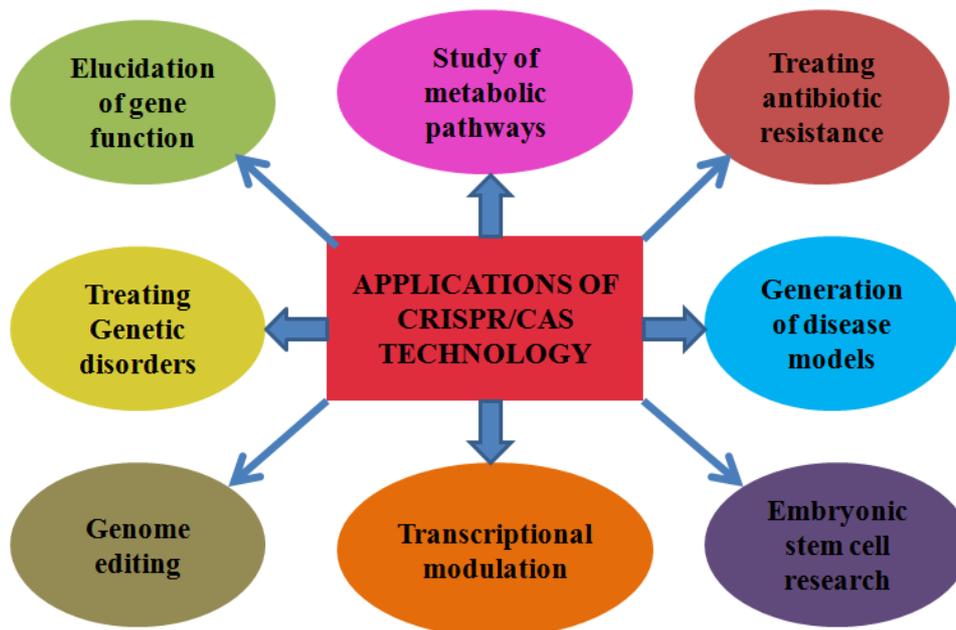


Fig 3: Scope of CRISPR-Cas technology in Aquaculture Domain

### 5.2 Applications of the CRISPR- Cas technology in fish models:

- **Zebrafish (*Danio rerio*)-** The CRISPR/Cas genome editing platform successfully introduced site specific heritable insertion/deletion (indel) mutations in endogenous zebrafish genes with 100% germline transmission efficacies at the target locus and mutation frequencies between 24-59% at 8 out of 10 tested genes [64]. The RNA-guided Cas9 nuclease efficiently generates site-specific somatic mutations (~35%) during target of *etsrp*, *gata4* or *gata5* locus in zebrafish embryos in vivo, biallelic conversion of *etsrp* and *gata5* in the resulting somatic cells in ~30% of embryos and generation of double-stranded breaks (DSBs) to induce homology directed repair (HDR) at or near the break site in the embryos [65]. Another finding elaborated the phenotypic rescue used by CRISPR/Cas system to target and repair a premature stop codon at the albino (*alb*) locus with high level of efficiency and precision using circular donor DNA of less than 1 kb in length flanked by CRISPR target sites. Here germ line transmission of functional targeted knock-ins of protein tags or of SNP exchange has been reported underscoring its suitability as a model for genetic research [66].

Multiple heritable genome modifications using golden/gol and tyrosinase/tyr (pigment formation) and *slpr2* and *spns2* (cardiac development) was demonstrated with insertion and/or deletion (indel) mutations introduced by the co-injection of multiple guide RNAs (gRNAs) and the nuclease Cas9 mRNA. This was the first report to show the germ-line transmission of the simultaneous multiple targeted genes disruption and the targeted chromosomal deletion in zebrafish [67]. Germline transmission data from 162 loci targeting 83 genes produced 99% success rate for generating mutations and average transmission rate of 28% in zebrafish genome facilitating efficient multiplexed gene targeting [68].

Another report confirmed the effective generation of knock-in transgenic zebrafish that have cell-type specific Gal4 or reporter gene expression via homology-independent repair using CRISPR/Cas9 system. This is the first report stating the generation of stable transgenic zebrafish in which large DNA constructs were knocked in for four endogenous genomic loci at a frequency exceeding 25% using this approach [69].

Successful heritable precise targeted gene knock-in by integration of exogenous *mCherry* or *eGFP* gene was also achieved into targeted genes (*tyrosinase* and *krttl1c19e*) in frame in zebrafish [70]. Other studies has shown a limited off-target mutation frequency produced by the prokaryotic

CRISPR/Cas9 type II genome editing system demonstrating heritable mutagenesis efficiency of about 86%. Knock-in efficiencies and mutation rates at potential off-target sites was found to be 3.5-15.6% and 1.1-2.5% respectively making it a powerful and highly efficient tool for in vivo studies [71].

- **Chinook salmon (*Oncorhynchus tshawytscha*)-** Genome editing in Chinook salmon embryos cell line (CHSE) was studied to overexpress monomeric form of Enhanced green fluorescent protein (EGFP) (cell line CHSE-E Geneticin resistant) and nCas9n, a nuclear version of Cas9 (cell line CHSE-EC, Hygromycin and Geneticin resistant) by knockout of the integrated EGFP gene. The EGFP gene was disrupted in 34.6% of cells and inactivation of the gene by deletions in the expected site was validated in 25% of clones exposing new avenues for cost-effective functional genomics studies compatible with high-throughput sequencing [72].
- **Atlantic salmon (*Salmo salar* L.)-** CRISPR/Cas9 system was used to target two genes involved in pigmentation, tyrosinase (tyr) and solute carrier family 45, member 2 (slc45a2) in embryos. About 40 and 22% of induced mutations for both CRISPRslc45a2/Cas9 and CRISPRtyr/Cas9 were found respectively in embryos at the 17 somite stage. Furthermore graded phenotype ranging from lack of pigmentation to partial loss and normal pigmentation was visible at hatching stage for both targeted genes. Targeted double-allelic knockout in the F0 generation was obtained providing evidence for investigating other important traits for the aquaculture industry [73].
- **Medaka (*Oryzias latipes*)-** Successful targeted mutagenesis at genomic sequence on the DJ-1 gene in embryos, injected with the single guide RNA (sgRNA) transcribed by a T7 promoter and capped RNA encoding a Cas9 nuclease, has been generated. This RNA-guided endonuclease (RGEN) technique has emerged as a potentially robust, efficient and flexible tool for genome editing in medaka [74].
- **The African turquoise killifish (*Nothobranchius furzeri*)-** This is the latest model and has practically many advantages due to low maintenance cost, rapid production of offspring and its relatively low span (4-6 months) and has been used exclusively for modelling age and age-related diseases *in vivo* [75, 76].
- **Tilapia (*Tilapia mossambica*)-** Highly efficient gene targeting was achieved by disrupting selected genes (nanos2, nanos3, dmrt1, and foxl2) and frameshift deletion mutations in foxl2 and dmrt1 induced by CRISPR/Cas9 were efficiently transmitted to the F1 generation indicating the gene disruption to be heritable. After mutation of germ cell or somatic cell-specific genes, obvious phenotypes were observed in the G0 generation implying its utility in generating loss-of-function mutants applicable in other teleost species as well [77].
- **Rohu (*Labeo rohita*)-** Using the CRISPR/Cas9 system in *Labeo rohita* (rohu), successful disruption of Toll-like receptor 22 (TLR22) gene, involved in innate immunity was demonstrated. The null mutant, arising from the integration of donor DNA via homologous recombination (HR), lacked TLR22 mRNA expression [78].

## 6. Conclusion

CRISPR-Cas systems is highly potent and can provide an

alternative effective strategy to selectively and quantitatively remove individual bacterial strains based purely on sequence information, imparting opportunities in the treatment of multidrug-resistant infections and the study of microbial consortia. The usage of *smart bomb* has not reached the aquaculture sector and if proper focus will be bestowed on developing smart bombs for particular antibiotic resistant pathogenic bacteria that brings about huge losses and is a menace to the fisheries sector, then definitely the problem of antibiotic resistance will be encountered.

These Smart bombs will selectively target only the pathogenic bacteria from a pool of microorganisms without affecting the beneficial microbiota. It will also be the best alternative to the antibiotics used that at a long run induces antibiotic resistance among the bacterial populations. This would undoubtedly transform biological research and spur the development of other novel and alternative therapeutic approaches for treatment of various infectious and diseases imparting innumerable applications in aquaculture sector. This approach could remarkably open new avenues for the development of “smart” antibiotics that circumvent multidrug resistance and differentiate between pathogenic and beneficial microorganisms.

## 7. Future Prospective

By targeting specific DNA strands through the CRISPR-Cas system the antibiotic resistance mechanism can be elucidated localizing the expanding resistance problem by specifically eliminating the whole target population and targeting disease at the molecular level. Similarly, targeting of the exponential rise of antibiotic resistant bacteria responsible for deteriorating conditions in aquatic animal's health could be done by precisely targeting the genes imparting disease for editing to make fish less susceptible to a specific frequently occurring disease leading to mortality. Again, knockout of the individual disease-related gene expression imparting resistance at the gene level with minimal off-target effects will also be facilitated.

Targeted knockout of genes using CRISPR-Cas9-mediated genome-editing represents a powerful approach to study gene function and to discern molecular mechanisms underlying complex human diseases thus signifying tremendous therapeutic potential and exploitation. Development of mutant models of fish by insertion or deletions of genes through the CRISPR/Cas technology would spur a revolution of enriching basic understanding of participatory distinctive roles being played by the targeted genes, potentially in the area of genome editing and transgenic research. CRISPR-Cas showers therapeutic potential for use in the correction of disease-causing genetic mutations and immense contributions to disease research and therapeutics will remain an exciting area of research to follow for the foreseeable future.

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