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RESEARCH ARTICLE OPEN ACCESS

Recoding the Codon Usage of *fis* Gene of *Pasteurella multocida* with Rare Codons Reduces Pathogenicity

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Abstract

Study was undertaken to evaluate gene recoding as an approach for vaccine development. *Fis* a transcriptional regulatory protein regulates the expression of more than 200 genes in different growth phases and at least 42 genes during exponential growth phase in *Pasteurella multocida*. We recoded this gene by replacing the codons with synonymous rare codons, without changing the amino acid sequence, to study its effect on growth and virulence in *P. multicoda*. The recoding of *fis* gene resulted in compromised competitive growth between recoded and wild strain bacteria in nutrient depletion condition. The bacterial uptake within murine peritoneal macrophages and adherence to Madin-Darby Bovine Kidney cells (MDBK) were significantly reduced ($P < 0.05$) but there was no difference in complement mediated killing between the recoded and wild strains. The Western blot had shown 2.08-fold reduction in the expression of *fis* protein in the mutant strain as compared to the wild strain *In vivo* pathogenicity study in mice showed one log decrease in LD_{50} of recoded strain. The results indicate that recoding of a key metabolic regulator is a viable option for attenuation of pathogenicity.

Keywords: Genome Recoding, Rare Codons, Pathogenicity, Virulence, Vaccine, *P. multocida*

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INTRODUCTION

Pasteurella multocida is a gram-negative, nonmotile coccobacillus generally found as a commensal in the upper respiratory tract of animals.[1] It affects a wide range of animal hosts such as cattle, buffalo, sheep, goats, pigs, and camels as well as wild animal and poultry.[1,2] It is the causative agent of haemorrhagic septicaemia (HS), one of the most acute and fatal diseases of cattle and buffaloes in Asia and Africa, causing huge economic losses to livestock industry.[2,3] The disease is caused by *P. multocida* serotype B:2 in Asia while serotype E:2 is responsible for this disease in African countries.[2,3]

Vaccination is the effective method for controlling HS in field condition. The existing vaccines is/are not very effective, require repeated administration at short intervals.[4] A live vaccine can combat this problem; the ideal live vaccine for HS should imitate the natural infection and be capable of surviving for a period of time within the host to stimulate protective immunity. Ideally, it should be a natural mutant or should contain a genetically-defined mutation(s) which renders the vaccine strain unable to establish continued growth *in vivo* and which minimizes the risk of reversion to the wild-type.[5]

The live-attenuated bacterial vaccines are mostly made by knocking out virulence, toxin or other genes of interest.[6] However, majority of the knockout live-attenuated vaccines are hyper attenuated, thus get cleared from the system before inducing an appropriate immune response.[5]

Many new ideas are currently being tried for attenuation of pathogens.[6] In this study, we have taken one such novel approach-codon bias. There are 20 amino acids, but 64 codons, three of which are stop codons resulting in multiple codons for most of amino acids. Multiple codons (synonymous) for an amino acids (degeneracy) of an organism are not used equally. Different

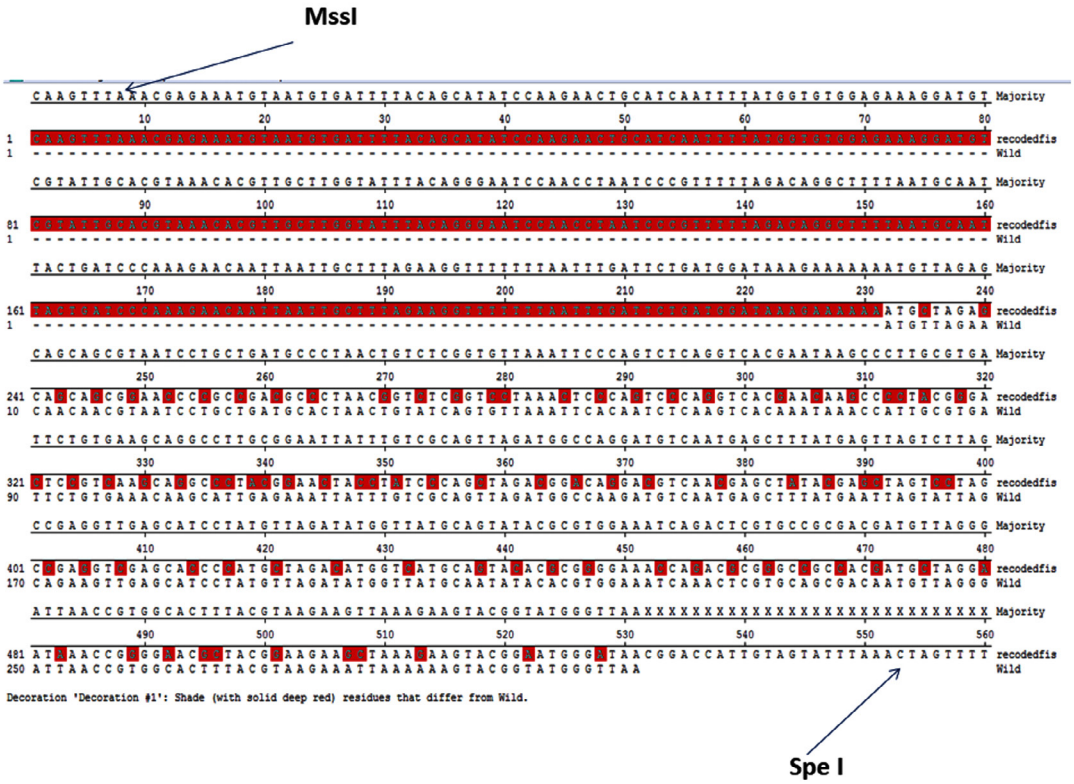

Figure 1. The sequence of synthetic recoded gene constructs with flanking regions. The flanking sequence, restriction sites and recoded sequences are marked

synonymous codons are used with different frequencies.[7] In general, highly expressed proteins use codons which are used more frequently and less expressed proteins use rarely used codons (rare codons or rare codon pairs) more frequently.[8] It has been surmised that replacing highly used codon with rare codons or rare codon pairs in the gene of a protein (virulence specific) may reduce its expression and thus compromise its virulence.[9] This attenuation method was first reported in polio virus[10,11] and further reported in many other viruses. There are only two reports of its use in bacteria, pneumolysin gene was recorded in *Streptococcus pneumoniae*[12] and *hfq* gene was recoded by us in *Salmonella Typhimurium.*[13] Both reports shown that recoding of target gene reduces pathogenicity of the bacteria. Here, we now further confirm that in *P. multocida* with *fis*

Table 1. List of Plasmids made

Plasmid	Relevant characteristics	Reference or source
pUC57FS	pUC57 + 560 bp insert including 300 bp recoded fis gene	Synthesized through GenScript
pDS132F	pDS132 containing 2.3 kp fragment from *Pasteurella multocida*	Made in this study
pUC57FS		
pDS132FM	pDS132 containing 560 bp insert from pUC57FS	Made in this study

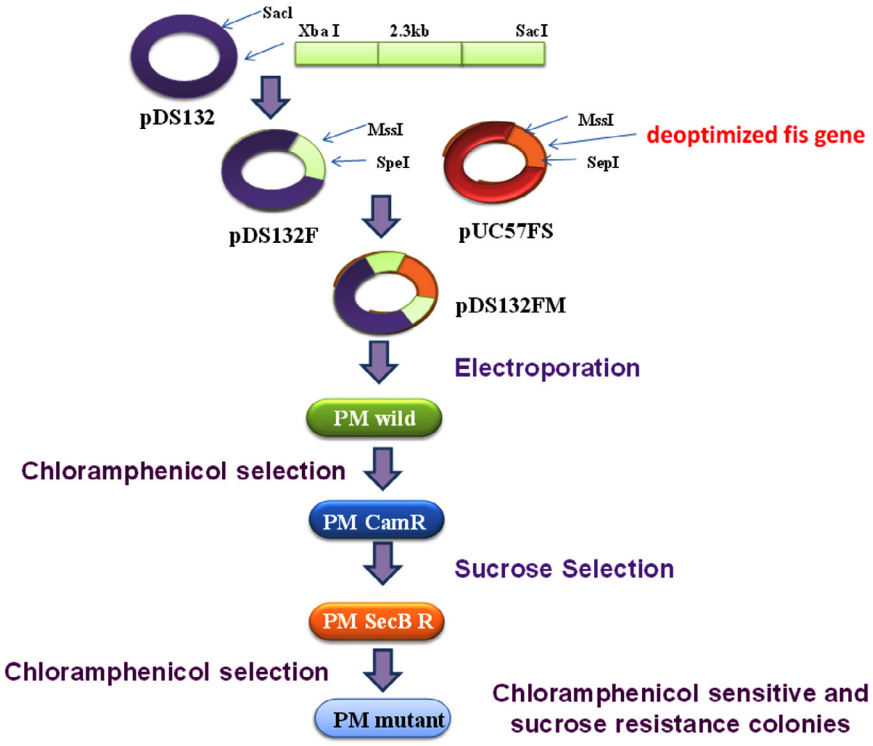

Figure 2. The strategy used for generating homologous recombinant. Legend: 2.3 kb region of *P. multocida* cloned into pDS132 (pDS132F) comprised 1 kb flanking regions covering 300 bp targeted region for recoding. Recoded region (300 bp) containing unrecoded flanking region with unique Mss I and Spe I in flanking region was custom made through vendors and was provided in pUC57 FS and was directionally cloned into Mss I, Spe I site of pDS132F. The resultant plasmid (pDS 132FM) was electroporated into *P. multocida*, the chloroamphenicol resistant clones (PM camR) were next sucrose selected. From the resultant colonies chloroamphenicol sensitive and sucrose resistant (CamS SucR) colonies were picked up and screened for the presence of recoded gene

gene. Fis protein was recognized as a factor for DNA inversion in the Hin and Gin family of DNA recombinases.[14] It is a key transcription regulatory protein that regulates more than 200 genes which include genes for the production of capsule and various virulence factors.[15,16]

MATERIALS AND METHODS

Bacterial strains, plasmids, primers and culture media

P. multocida P52 strain used in this study was provided by Division of Standardization, Indian Veterinary Research Institute, Bareilly, U.P.,

Table 2. List of Primers Used

Name	Sequence (5'-3')	RE Site	Accession No. - AE004439.1 and position
FLN1	AGCTATCTAGATCACCGCCGCGTTATCTTTA	XbaI	1281328-1281347
FLN2	ATCGAGAGCTCCTTATCGGTCCGGGTATGTT	SacI	1283682-1283663
FSC1	TCTGGTTTCCCCGCGTGTAC*		
FSC2	TCCCAGTCCCAGGTCACGAA*		

*Primer design from recoded fis sequence

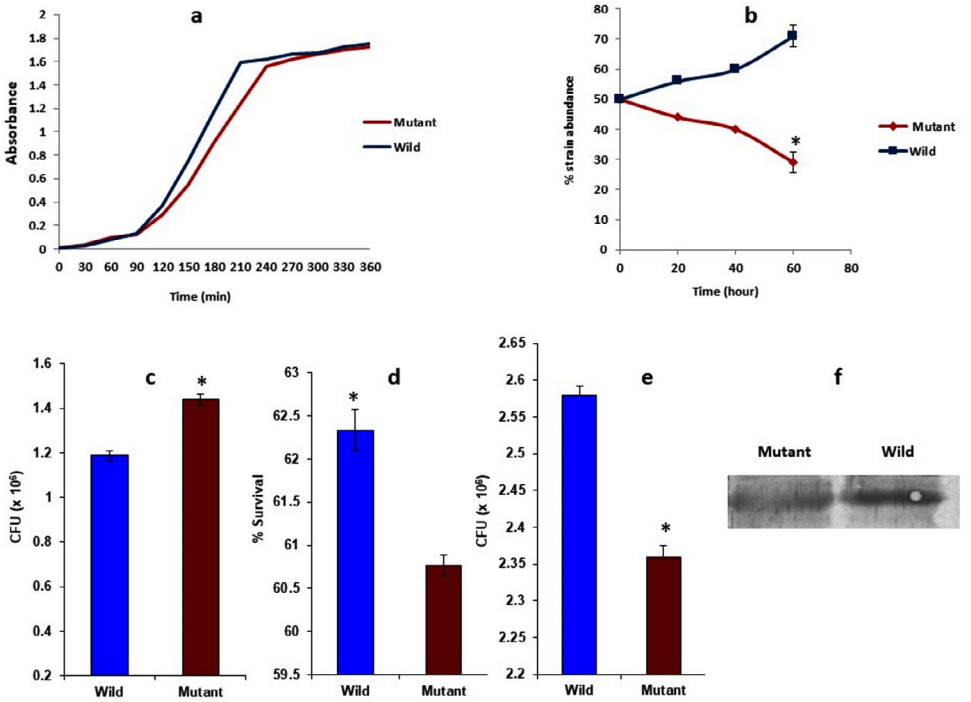

Figure 3. (A) *In vitro* growth kinetics (exponential growth curve) of wild and mutant strain. (B) Growth competition between wild and mutant strains. Co-culturing equal inocula of wild and mutant. (C) Macrophage uptake assay of wild and mutant strain. Results showing the numbers of surviving bacteria released from lysed macrophages. (D) Bactericidal assay of wild and mutant. Results showing % survival of bacteria after complement mediated killing. (E) Adherence of wild and mutant to MDBK cells. Results showing numbers of bacteria adhere to MDBK cells. (F) Expression of fis protein in mutant and wild strain

India. The bacteria strain *E. coli* DH5α-λ pir bacteria strain used in this study was obtained from Dr. Andrew Camilli, Tufts University, Boston. Plasmids made and used in this study are listed in Table 1. pDS132 plasmid was obtained from Dominique Schneider, Universite Joseph Fourier, France. Primers used in this study are listed in Table 2. *P. multocida* strains were grown routinely in brain heart infusion (BHI) broth (High Media, India) or on BHI agar containing 5% sheep blood. When required, the media were supplemented with chloramphenicol (High Media, India) @30 µg/ml.

Design and construction of recoded *fis* gene of *P. multocida*

The recoding of *fis* gene was performed by substituting the codons with codons least used in *P. multocida,* which were identified from the codon usage pattern obtained from http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=272843. The recoded gene (Figure 1) with flanking region (231 bp upstream, 29 bp downstream) containing unique restriction sites (MssI, SpeI) was synthesized through vendor and was provided as a cloned product in pUC57.

Molecular biology techniques

GeneJet plasmid Miniprep kit (Fermentas, India) was used for isolation of plasmid DNA. Enzyme used for restriction digestion and ligation were obtained from Fermentas, India. Recombinant DNA was incorporated into *P. multocida* by electroporation technique using MicroPulser (Bio-Rad, India) as previously described.[17,18] DNA was amplified by PCR with the help of High fidelity DNA polymerase (Roche) and PCR amplified products were gel purified using PureLink quick gel extraction kit (Invitrogen, India). The sequencing was done through vendors (Bioserve Biotechnologies, India)

Generation of mutant *P. multocida*

We first amplified the 2.3 kb region of *P. multocida* comprising *fis* gene (300 bp) and 1 kb flanking regions, subsequently cloned into plasmid pDS132 (Accession No. AY489048) by directional cloning. The *fis* gene from resultant plasmid was replaced with the recoded *fis* gene using compatible restriction enzymes and electroporated into *P. multocida.* The strategy for generating homologous recombinant is shown in Figure 2. The single crossovers were selected on chloramphenicol and double crossovers were selected on sucrose. The sucrose resistant and chloramphenicol sensitive colonies were screened by PCR. The insertion of recoded gene and its orientation was confirmed by sequencing. Details about generation of mutant *P. multocida* is described in the supplemented document.

Western blot detection of *fis*

Recombinant *fis* protein with Freund's complete adjuvant (FCA) was inoculated in rabbit for rising of hyperimmune sera on 1st, 14th, 21st and 28th day. Western blot was performed for conformation of *fis* antibody in serum. Western blot was performed in both wild and mutant *P. multocida* as described previously.[12] The density of blot was recorded by using Quantity one software (Biorad).

***In vitro* growth kinetics and growth competition assay**

P. multocida single colony was picked up from the plate and inoculated into 5 ml of BHI broth and incubated at 37 °C with constant shaking at 180 RPM overnight and 50 µl of this culture was inoculated into 50 ml BHI broth. Then culture was incubated at 37 °C with constant shaking and OD_{600} was measured at 30 min interval for 6 hrs. Growth competition assay was performed by method described erlier.[19] The number of mutant and wild strains were counted by randomly picking of bacteria followed by colony PCR.

Bactericidal assay

Bactericidal assay was carried as described previously.[20] Data were recorded as % survival in 50% active normal bovine serum (aNBS) compared to that in heat-inactivated bovine serum (iBS).

$$\% \text{ Survival} = (\text{CFU in aNBS} / \text{CFU in iNBS}) \times 100$$

Macrophage uptake assay

Mouse peritoneal macrophages were harvested as described previously.[21] The mice were stimulated by intraperitoneal inoculation with 2 ml of 10% bacterial peptone 72 h prior to harvest. Peritoneal cavity was washed with 8 ml of RPMI 1640 (Gibco, india) supplemented with 5% fetal

calf serum (Genetix, India), and 5 U/ml heparin to harvest the macrophage and finally resuspended in 3 ml RPMI 1640 media. Macrophages were seeded into a 24 well plate (Nunc, Denmark) at a concentration of 10^5 cells per well 12 hour prior to infection. Mid log phase grown (OD 0.4-0.6) wild and mutant strains of *P. multocida* were added to the macrophages in 1:100 macrophage: bacteria ratio and kept at 37 °C for a period of 90 min in a CO_2 incubator. All plates were washed three times with PBS and resuspended in RPMI media containing 50 µg/ml gentamicin (Himedia, India), followed incubated at 37 °C for 90 min again. There after supernatant was removed, and lysis of the macrophages were performed by using 0.01% Triton X 100 (Sigma, USA) and serial dilutions were plated to determine the CFU.

Adherence assays

Adherence of bacteria to Madin-Darby Bovine Kidney (MDBK) cells (National centre for cell science, Pune, India) was measured quantitatively as describe by Letourneau *et al*.[22] Briefly, MDBK cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% foetal bovine serum, 100 units of penicillin/ml and 100 µg of streptomycin/ml in 24 well plate and incubated at 37 °C in a 5% CO_2 humidified incubator until confluence. Plates were washed thrice with PBS. DMEM Supplemented with 10% foetal bovine serum was added to each well. Mid log phase grown *Pasteurella multocida* was centrifuged and resuspended in DMEM and the OD_{600} was adjusted to 1. 5 µl of bacterial suspension was added in triplicate to individual wells and then the plates were kept on dancing shaker for 10 min, after which the plates were incubated for 2 h at 37 °C. The wells were then washed three times with PBS medium to remove nonadherent bacteria. After nonadherent cells were removed by washing, 0.5 ml of trypsin solution was added to each well, and the plate was incubated for 5 min at room temperature. Then, the contents of each well were suspended by repeated pipetting; serial (10-fold) dilutions of the suspension were spread onto BHI plate, which were incubated for 16 hrs. Plates containing between 30 and 300 colonies were counted to determine the number of adherent bacteria.

Assessment of virulence in mice

Swiss Albino female mice (20-25 g) of six to eight week-old age obtained from the Laboratory Animal Resource (LAR), Indian Veterinary Research Institute, Izatnagar (U.P.), India, for assessment of virulence in mice. All animal experiments were permitted by Institute Animal Ethics Committee and CPCSEA (Committee for the purpose of control and supervision of experiment on animals), Ministry of environment and Forestry, Government of India. The animals were acclimatized for one week before the start of the experiment. *P. multocida* strains were grown in BHI broth for 12 hrs at 37 °C and then the cells were pelleted and suspended in PBS and OD_{600} was adjusted to 1. Then 100 µl containing, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 CFU were administered intraperitoneally (IP) into 6 groups (each containing 5 mice) respectively and 50% lethal dose (LD_{50}) was determined.[23]

Statistical analysis

The analysis of *in vitro* data were carried out by Student's ''t'' test using statistical programme SAS. 50% lethal dose (LD_{50}) was calculated.[23] Results were determined to be statistically significant at a 95% confidence level.

RESULTS AND DISCUSSION

P. multocida acts as etiological agent of may important diseases of animal origin such as fowl cholera in poultry and haemorrhagic septicaemia in cattle and buffaloes. Fis is a transcriptional regulatory protein and controls expression of more than 42 genes in *P. multocida*.[17,24] In this study we created a *fis* mutant of *P. multocida* P52 by homologous recombination (Figure 2). The *fis* mutant we created had identical ORF (open reading frame) to that of parent strain, the difference was use of rare synonomus codons. Two similar studies had been reported in bacteria *S. pneumoniae*[12] and *S. Typhimurium*.[13,14] We have used rare synonomus codons (codon bias) as used by us formerly in *S. Typhimurium*[13,14] while other worker used codon pair bias for recoding in *S. pneumonia.*[12] Another difference was in the choice of the gene.

The *fis* gene product regulates from 42 to more than 200 genes at various growth phase[15] in

different species. The *fis* deletion mutant shows defective growth during exponential phase in *Haemophilus ducreyi,*[25] *E. coli,*[26,27] and in *Legionella pneumophila*.[28] Likewise, our study shows difference in growth kinetics between mutant and wild strain of *P. multocida* (Figure 3 A) consistently, however, the difference was not significant when the slope of exponential growth curve slope of parent (0.0061 ± 0.00033) and the mutant strains (0.0056 ± 0.00026) was compared. In growth competitive assay the wild strain out competed (2.4:1) mutant strain significantly ($P < 0.05$) (Figure 3 B) in nutrient depletion condition. The nutrient rich medium does not reflect *in vivo* conditions. Nutrient depletion condition also does not reflect truly the *in vivo* conditions; however, it is more akin to *in vivo* conditions. Our data show that in nutrient depletion condition the mutant's fitness to compete with the wild strain is significantly compromised.

The ability of pathogen to overcome host response is reflected in its ability to avoid uptake by macrophages.[21] Our results on macrophage uptake of mutant and wild strain show significant difference in bacteria uptake ($P < 0.05$) (Figure 3 C). The uptake of mutant was high as compared to wild strain indicating their high susceptibility to phagocytosis. Nevertheless, we did not find any difference in complement mediated killing between mutant and wild strain (Figure 3 D). The *fis* protein enhances the invasion and intracellular replication ability of *Salmonella enterica* within macrophage cells.[29] One of the many pathways *fis* affects is capsule biosynthesis[15] and capsule inhibits phagocytic uptake, however capsule has no role in complement mediated killing.[30] We have not studied capsule biosynthesis and so cannot say with authenticity that capsule biosynthesis is responsible for these results. The *fis* controls large number of genes that include capsule biosynthesis so there could be other factors or multiple factors acting in concert; however, our concern was to study the phagocytic uptake not the mechanism by which this effect takes place. The adherence of bacteria to eukaryotic cells is another parameter in the study of virulence.[25] Again we found significant reduction in adherence to eukaryotic cells in mutant as compared to wild strain (Figure 3 E). The *flp* gene expression is required for adhesion of *H. ducreyi* and *flp* gene expression is regulated by *fis.*[25] Again it is difficult to deduct based on past results that it is *flp* acting in concert with *fis* in reducing adherence because *fis* has multiple roles as a transcription regulator. However, our results show indirectly that this could be the reason for decrease in adherence. To assess the *fis* protein expression in mutant and wild strain we did Western blot of bacterial lysate of mutant and wild strain (Figure 3 F). We did not use an internal protein for normalization because we were not sure which protein to use since *fis* being a transcriptional regulator controls large number of proteins. We normalized our data with protein concentration (loaded equal amount of protein). The Western blot data shown that 2.08 fold decline in *fis* protein expression in the mutant strain as compared to the wild strain. The difference in the *fis* expression is substantial even if take into account the experimental error in measuring protein concentration, loading and densitometer analysis.

Most of the data we had studied indicated a decrease in parameters related to pathogenicity. When the experiment was done to assess lethality *in vivo* in mice, we found a decrease of ten fold in mutant as compared to wild. Our data on decrease in pathogenicity after recoding is broadly in accord with viruse[15] and for *S. pneumoniae*[12] and *S. Typhimurium*.[13] In most cases of recoding experiments in viruses whole genome recoding has been done which is not possible for bacteria because of genome size. In *S. pneumoniae* pneumolysin gene was recoded. The results of that study [12] show decrease in pathogenicity (50% survivability of mice post infection). We also got similar results, though the data are not strictly comparable (survival versus lethal dose).

CONCLUSION

The data suggest that increasing the level of recoding by increasing the number of genes targeted for deoptimization may decrease the pathogenicity to desire extent. A graded decrease in the pathogenicity is possible by using only non-toxin genes. We feel that this concept of using key metabolic regulators as a target for recoding may

be useful for making live attenuated vaccine for bacterial pathogens.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.19.2.49

Additional file: Supplementary documented.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Animal Ethics Committee (IAEC) of the Indian Veterinary Research Institute (IVRI) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. All experimental procedures were conducted in strict accordance with the ethical standards and guidelines prescribed by the CPCSEA.

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