Original Article

J Nepal Health Res Counc 2021 Jan-Mar;19(50): 76-82

DOI: https://doi.org/10.33314/jnhrc.v19i1.3282

Therapeutic Efficacy of Bacteriophage Therapy to Treat Carbapenem Resistant *Klebsiella Pneumoniae* in Mouse Model

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ABSTRACT

Background: Global emergence of carbapenem-resistant *Klebsiella pneumoniae* is a major public health concern. Phage therapy – application of lytic phage to kill pathogenic bacteria – is considered as one of the promising alternatives to tackle this antibiotic crisis in recent days. This study aimed to isolate, characterize and evaluate therapeutic efficacy of a novel *K. pneumoniae* phage in mouse model.

Methods: A novel lytic bacteriophage (phage) Kp_Pokalde_002 was isolated against carbapenem-resistant *K. pneumoniae* (Kp56) and characterized. Safety parameters of the phage were evaluated by bioinformatic analysis of its genome. A lethal dose (~1×10^7 CFU/mouse) of Kp56 was determined and administrated in the mice. The infected mice were treated with phage Kp_Pokalde_002 at a multiplicity of infection (MOI) 1.0 (~1×10^7 PFU/mouse) via both oral and intraperitoneal (IP) routes.

Results: Isolated phage comprised an icosahedral capsid with a short tail. Based on genome analysis, the phage was strictly lytic belonging the Podoviridae family (T7-like viruses) and free from any virulent and antibiotic-resistant genes. The phage was stable up to 60 °C for 30 minutes and effective between pH 4 to 11 (optimum pH 9). The phage exhibited a short latent period (20 minutes) with burst size of 121 phage particles per infected cell. The infected mice were rescued with the phage therapy via both oral and IP route. Significant reduction of bacterial load (3-7 $\log_{10}$ CFU/ml) in the blood and lung was observed in the treatment group.

Conclusions: We provide an evidence of successful phage therapy against carbapenem-resistant *K. pneumoniae* infected mouse model using locally isolated lytic phage.

Keywords: Bacteriophage; *klebsiella pneumonia*; phage therapy

INTRODUCTION

Klebsiella pneumoniae is one of the opportunistic pathogens, often seen as multidrug resistant including 4th generations cephalosporins. It causes a wide variety of nosocomial infections with high morbidity and mortality.[1] Due to difficulty in treating infections caused by these superbugs, an alternative approach must be sought. Among many alternatives, bacteriophage-mediated treatment or phage therapy is promising.[2] Phage therapy in animal models has demonstrated successful outcomes against multiple pathogens such as extended spectrum beta-lactamase (ESBL) and carbapenem-resistant bacteria like *Escherichia coli, Acinetobacter baumannii, K. pneumoniae and Pseudomonas aeruginosa*.[3-6]

Major challenges of phage therapy are poor understanding of phage-host dynamics within the body including phage availability, stability and accessibility to the target host, development of bacteriophage resistant mutants, immune mediated phage neutralization and uncertain route and dose of administration.[7] Till date, only few studies have been conducted to evaluate the routes of phage administration to treat systemic infection caused by carbapenem-resistant *K. pneumoniae*. Thus, this study aimed to isolate, characterize and evaluate therapeutic efficacy of bacteriophage to treat carbapenem-resistant *K. pneumoniae* in mouse model using locally isolated phage.

METHODS

The study was conducted at the Central Department of Biotechnology (CDBT), Tribhuvan University, Nepal. Phage genome sequencing and electron microscopy was performed in Xcelris Labs, Ahmedabad and Jawaharlal

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Nehru University, New Delhi, India. A clinical isolate of *K. pneumoniae* (Kp56) which was molecularly confirmed as a carbapenem-resistant strain from previous study, was obtained from the Microbiology Laboratory, CDBT (unpublished data). The bacterial strain was propagated in Luria-Bertani (LB) broth (HiMedia, India) at 37 ºC.

A novel phage Kp_Pokalde_002 was isolated using the Kp56 as a host from the municipal wastewater canal, Kathmandu. Standard double layer agar assay (DLAA) was used for the isolation and propagation of the phage as described elsewhere.[8] Phages were further purified by isopycnic CsCl density-gradient ultracentrifugation described previously.[9] The phage preparation was passed through 0.22micron, syringe filter (Whatman, Sigma Aldrich, USA) and stored at 4 °C until further use. For transmission electron microscopy (TEM), the purified phage lysate was fixed with 2% paraformaldehyde and 2.5 % glutaraldehyde. Two microliters of the fixed phage lysate was spread on a carbon-coated copper grid and negatively stained with 2.0 µl of 2% (w/v) uranyl acetate (pH 4.5). The copper grid was dried completely and examined under JEM-2100F transmission electron microscope (JEOL, Japan) at 200 kV field emission.

Stability of the phage Kp_Pokalde_002 at different temperatures and pHs was determined as described previously with modifications.[10] Briefly, for temperature stability, 1.0 ml phage lysate of 10^8 PFU/ml in SM buffer (100 mM sodium chloride, 10 mM magnesium sulphate, 50 mM Tris-HCl, pH 7.5 and 0.01% (w/v) gelatin) was aliquoted in an Eppendorf tube. The aliquots were incubated at 25 °C, 37 °C, 50 °C, 60 °C and 70 °C for up to 180 minutes and titrated using DLAA. Stability of the phage at different pHs was assessed by mixing phage lysate (10^8 PFU/ml) in SM buffer (pH 2-12) at a ratio of 1:10 (v/v) and incubated at 37 °C for 1 hour. The phage suspension was then titrated using DLAA. One-step growth experiment was performed to determine the burst size as described previously with modifications.[11] Briefly, 100 µl of exponentially growing Kp56 (OD_{600} = 1.0) in LB broth was mixed with 100 µL of phage lysate at MOI 1.0 and incubated (without shaking) at 37 °C for 10 minutes for adsorption. The mixture was centrifuged at 11,000 rpm at 4°C for 10 minutes and supernatant was discarded to remove un-adsorbed phage particles. The pellet was resuspended in 10 ml of LB broth and incubated at 37 °C. Aliquots of 0.1 ml were taken at intervals of 5 minutes for up to 70 minutes and its titer was estimated using DLAA. The burst-size was calculated as a ratio of the number of phage particles liberated with the initial number of infected bacterial cells.

The genomic DNA of the phage Kp_Pokalde_002 was extracted using the phenol-chloroform method described earlier.[12] Whole-genome sequencing was performed using Illumina Nextseq500 platform. The DNA library was prepared using an Illumina Nextera XT kit. The de novo sequence assembly was performed using SPAdes 3.13.1.[13] Structural and functional annotations of the assembled contigs were performed using tools available at the Galaxy and Web Apollo (https://cpt.tamu.edu/galaxy-pub/). The phage genome was screened for bacterial toxins, virulence factors and antimicrobial-resistant genes using Virulence Factors of Pathogenic Bacteria (VFDB) [14], Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca) and Resistance Gene Identifier (RGI, v5.1.0) databases with default parameters. The complete genome sequence and associated raw data of the phage Kp_Pokalde_002 are available under GenBank accession number MT425185, BioProject accession number PRJNA594990, and SRA accession number SRR11570037.

Six-eight weeks old female Swiss albino mice (23 ± 2.5 g) were purchased from the Natural Products Research Laboratory (NPRL), Kathmandu. All animal experiments followed the guidelines established by the Nepal Health Research Council, Kathmandu (ethical approval no.161/2018). Mice were anesthetized with chloroform before invasive procedures and euthanized by cervical dislocation if required. Mice were housed in the animal housing facility at the CDBT and fed with normal antibiotic-free diet. All mice were housed under identical conditions.

Minimum lethal dose (MLD) of Kp56 in mice was determined as previously described with modifications.[15] One milliliter of mid-log phase bacterial suspension in LB broth (OD_{600} = 1.0) was centrifuged at 4,000 rpm for 10 minutes and washed with 1.0 ml sterile phosphate buffer saline (PBS) three times. The bacterial pellet was resuspended in 1.0 ml normal saline and serially diluted to obtain bacterial count ~1×10^6, ~1×10^7, ~1×10^8 and ~1×10^9 CFU/ml. Thirty mice were divided into six groups. An aliquot of 200 µl of diluted bacterial cell suspensions (~1×10^5, ~1×10^6, ~1×10^7, ~1×10^8 and ~1×10^9 CFU/ml) was injected into each of the five group of the mice through IP route. Two hundred microliter of normal saline was injected into the sixth (control) group. The mice were observed for signs of illness and survivability for up to 7 days.

Efficacy of phage therapy in mouse model was evaluated as previously described with several modifications.[16-17] Briefly, mice were divided into seven groups (5 mice/group). A lethal dose (~1×10^7 CFU/mouse) of Kp56 was injected through IP route into four of the seven groups. First group was injected with 200 µl of SM buffer via IP

route as a sepsis-positive control. The second and third groups of mice were treated with a single dose of 200 µl of phage Kp_Pokalde_002 (1.2×10^8 PFU/ml) via either oral or IP route immediately after bacterial injection. Two hundred microliter of phage Kp_Pokalde_002 (1.2×10^8 PFU/ml) was injected through IP route after one-hour of bacterial challenge in the fourth group. In fifth group, 200 µl of phage Kp_Pokalde_002 (1.2×10^8 PFU/ml) was injected intraperitoneally 24 hours prior Kp56 infection (pre-phage therapy group) and sixth group was a vehicle control group which was injected with 200 µl of SM buffer only. The last, phage only group, mice were injected with 200 µl of phage Kp_Pokalde_002 (1.2×10^8 PFU/ml) via IP route. Bacterial count was enumerated from the blood and homogenized lung tissues samples. Animals were observed for their health conditions and survivability for 15 days.

For statistical analysis, data were expressed as a mean, standard deviation (SD) of the mean and analyzed under an ordinary one-way and two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test and student's t-test using GraphPad Prism (Version 8.3.0). For phage therapy, survival curves were compared for significance using the log-rank (Mantel-Cox) test. Differences with $p < 0.05$ were considered statistically significant. Each of the experiments was performed three times. The error bars in the graphs are representative of the standard deviation in each experiment.

RESULTS

Newly isolated phage (Kp_Pokalde_002) produced a large (8-10mm), round, clear plaques surrounded by a halo zone, indicating presence of depolymerase activity on the lawn of the host Kp56 (Figure: 01-A). On TEM, the phage Kp_Pokalde_002 was found to be a tailed phage consisting an icosahedral head measuring approximately 53 nm with a short non-contractile tail measuring approximately 13 nm in length (Figure: 01-B). According to the International Committee on Taxonomy of Viruses (ICTV) guidelines, the phage morphologically belongs to the Podoviridae family of the Caudovirales order.[18]

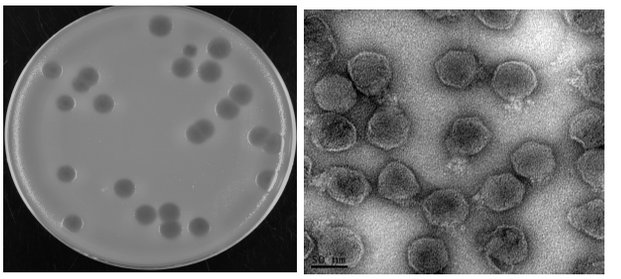

Figure 1. Morphological characterization of phage Kp_Pokalde_002.

Thermal stability of the phage Kp_Pokalde_002 was determined by incubating the phage lysate at different temperatures (25 °C, 37 °C, 50 °C, 60 °C and 70 °C) for up to 180 minutes. The phage titer did not significantly decrease ($p > 0.05$) at 25 °C and 37 °C for up to 180 minutes while the phage titer decreased rapidly after 60 minutes when incubating at 50 °C and completely lost its viability after 180 minutes (Figure 02-A). Likewise, the phage titer decreased significantly at or above 60 °C after 30 minutes. The phage viability was significantly unaffected at pH 6 to 9 ($p < 0.05$), while the phages remained viable between pH 3 to 11. The phage was completely inactivated below pH 2 and above pH 12 (Figure 02-B). The one-step growth curve showed the phage had a short latent period of 20 minutes. Similarly, the growth curve of the phage reached to the plateau at 50 minutes (Figure 02-C). The burst size was calculated based on the final titer of the phage and number of infected bacterial cells. The average burst size was found to be 121 phage particles per infected cell.

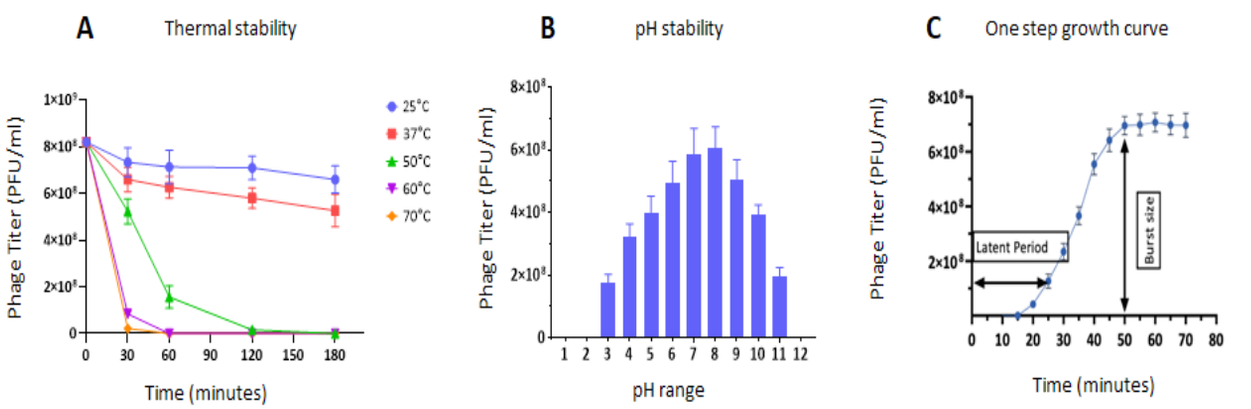

Figure 2. Thermal stability, pH stability and one step growth curve of phage Kp_Pokalde_002.

The complete genome of the phage Kp_Pokalde_002 was composed of a linear double stranded DNA of 41,816 bp in length with an average GC content of 53% (Figure: 03). The phage contained 180 bp direct terminal repeats at both ends. The phage genome comprised 45 open reading frames (ORF), one host RNAP promoter, and 12 phage promoters. Two rho-independent terminator sequences and no tRNA genes were predicted throughout the genome. All the predicted ORFs were located on the same forward strand of the DNA. Besides the predicted protein functions, the predicted amino acid size, the genomic position, the transcriptional orientation, and the GenBank protein identification numbers of the genome can be found in the GenBank accession number: MT425185. The genome of phage Kp_Pokalde_002 did not encode any known toxins and/or virulence factors (VFs) and antibiotic resistant genes.

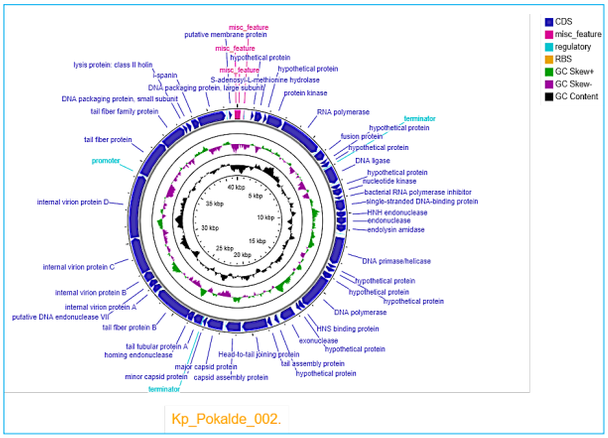

Figure 3. Circular genome view of the phage Kp_ Pokalde_002.

Minimum lethal dose (MLD) experiment showed that 100% mice died within 48 hours when injected with ~1×10^7 CFU/mouse of Kp56 intraperitoneally (Figure: 04). After the injections of ~1×10^6 CFU/mouse, the survival rate decreased to 60% and after the injections of ~1×10^5 CFU/mouse, the survival rate was 100%. Therefore, ~1× 10^7 CFU/mouse was considered as a lethal dose (LD_{100}) in Swiss albino mice.

For phage therapy, the mice were infected with the lethal dose of Kp56 and treated with the phage Kp_ Pokalde_002 through IP and oral route at MOI 1.0. Both concurrent and 1 hour delay intraperitoneally treated mice were rescued with the survival rate of 100% in contrast to the control group without phage therapy ($p < 0.05$). However, survivability of the oral-treated mice was decreased to 40% (Figure: 05). Interestingly, 80% mouse survivability was recorded in the pretreatment group ($p < 0.05$) where phage Kp_Pokalde_002 was administrated intraperitoneally 24 hours prior to the Kp56 infection.

Bacterial load was significantly reduced ($p < 0.05$) by 3-5 $\log_{10}$ CFU/ml at 8 hpi and 5-7 $\log_{10}$ CFU/ml at 24 hpi in the blood and 2.4 $\log_{10}$ CFU/ml at 8 hpi and 4-7 $\log_{10}$ CFU/ml at 24 hpi in lungs when treated with the phage. In contrast, bacterial load was lower in blood and lungs at any time point(s) when phage was administrated via IP route as compared to the oral route (Figure: 06). Normal control groups survived without any symptoms of illness for 15 days. Consistent results were obtained in three independent experiments.

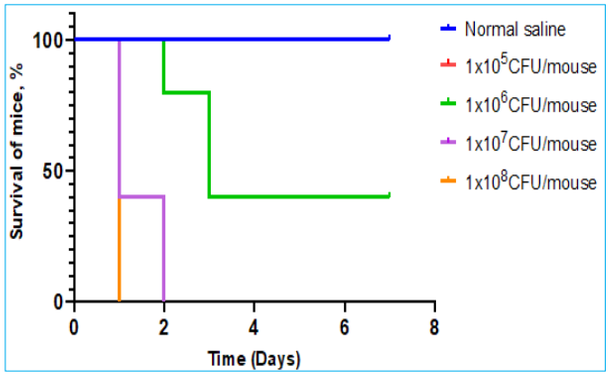

Figure 4. Determination of minimum lethal dose (MLD) of *K. pneumoniae* (Kp56).

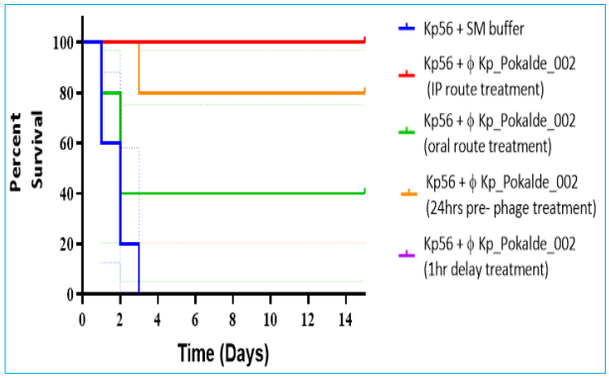

Figure 5. Efficiency of phage therapy in-vivo.

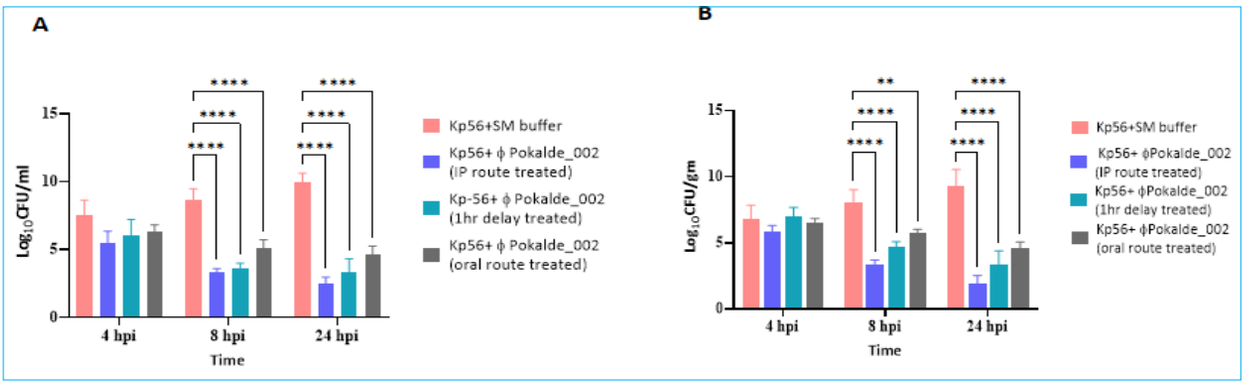

Figure 6. Bacterial load reduction by phage Pokalde_002 in blood and lung tissues.

DISCUSSION

Developing safe and effective phage therapy involves preparation of well-characterized phage library with complete information regarding its physiochemical and genomic properties to determine lysogenicity, antibiotic resistance and toxin/virulent genes.[19] In this study, the morphological and genomic organization of newly isolated phage Kp_Pokalde_002 indicates that the phage was strictly lytic and shares 96% of its genomic identity with the Klebsiella virus KP32 (Accession No: NC_047968) belonging to the genus of T7-like viruses, Podoviridae family. Based on bioinformatic analysis, genome of phage Pokalde_002 did not have any known antibiotic resistant genes. The phage formed a round, clear plaques with distinct peripheral halo indicating the phage has depolymerase enzyme responsible for degrading capsular exopolysaccharides and biofilm produced by *K. pneumoniae*.[20] The phage was found to be stable in wide range of thermal and pH range make it appropriate in varying external conditions during therapy. One step growth experiment showed the phage Kp_Pokalde_002 had a short latent period with high burst size indicating rapid multiplication and high number or progeny production which further infects the hosts in its vicinity thereby eliminating host (or pathogen) efficiently within short period and unlikely to develop phage resistant mutants.[21] Thus, we could clearly consider that the phage as a potential candidate for phage therapy.

In present study, Kp56 infected mice were rescued by a novel phage Kp_Pokalde_002 when administrated via both oral and IP route. Previous results have also demonstrated that both oral and IP routes of administration protected mice from death infected with *K. pneumoniae* and *P. aeruginosa*.[15,22] Watanabe et al. reported mouse survival rate was significantly higher in IP and IV routes of administration compared to the oral route.[23] Similarly, Hung et al. concluded that oral administration of phage was more efficient than IP route at protecting mice infected with *K. pneumoniae* during the initial infection period, while IP route showed therapeutic efficiency during the later stage of the infection.[15] Our result shown that the phage Kp_Pokalde_002 was able to cross the gut wall successfully into the blood and other tissues of mice. However, survivability of oral-treated mice was reduced to 40% as compare to IP route. The possible reason for this result may be due to orally treated phage might not have reached into the systemic circulation and localize into the infected site at an optimum concentration due to adverse environment of the gastrointestinal tract such as gastric acidity, presence of enzymes/bile juice and poor intestinal absorption rate of the phage.[24] It was shown that the oral route of phage delivery was efficient in the treatment of gastrointestinal infections.[25] Studies have shown that microencapsulation of phage protects them from the adverse gut environment and enhance the efficacy when administered orally.[26] Further, the phage Kp_Pokalde_002 was also able to rescue mice from the lethal infection significantly ($p < 0.05$) when administrated 24 hours prior bacterial challenge. This confirms that the phage has good pharmacokinetics property and is stable within the body of the mice for up to 24 hours.

Successful phage therapy depends on various factors like time of phage injection, MOI (phage to bacteria ratio), host immune response and phage clearance, phage burst size, phage half-life, and bacterial resistance in-vivo.[27] Interestingly, in our study, all of the mice were rescued from the infection in an immediate and delayed phage treatment group (1 hpi). Wang et al. reported that one hour delayed phage therapy led to 56% reduced animal survival while others reported that 100% of animals survived when the phage was administrated within 4 to 7 hours post infection. Similarly, 50% of animals survived when the phage was administered 24 hours post infection.[3,4,28,29] In addition, bacteria count significantly

decreased in the phage-treated mice, which suggests that the phage effectively eliminated the bacteria in-vivo. These studies demonstrated that the phage Kp_Pokalde_002 is a viable candidate for phage therapy against carbapenem resistant *K. pneumoniae* infections.

CONCLUSIONS

The results of this study provide a strong evidence of successful phage therapy in carbapenem-resistant *K. pneumoniae* infected mouse model. Based on physiochemical and genomic characters, the phage Kp_Pokalde_002 can be considered as safe therapeutic candidate. However, further research on pharmacokinetics and pharmacodynamics aspects of phage therapy and a library of well characterized phages is necessary before moving on phage therapy in human.

ACKNOWLEDGMENTS

This work was funded by the Ph.D. fellowship and research support awarded to Gunaraj Dhungana (award numbers PhD/73-74/S and T-07) by the University Grants Commission (UGC) in Nepal. Nepal Academy of Science and Technology (NAST), Prof. Dr. Krishna Das Manandhar, Head, Central Department of Biotechnology, TU, Xcelris Labs, Ahmedabad and Advanced Instrumentation Research Facility (AIRF), JNU, New Delhi.

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