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Isolation and Characterisation of *Bacillus* Phage from Equine Carcass Disposal Site

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ABSTRACT

Bacteriophages play an important role in bacterial control in natural niche however a little is known about *Bacillus* sp. phages prevailing in cadaver affected soils. In the current study, the *Bacillus* sp. phage was isolated from the equine cadaver disposal site and characterised to gain an insight into the issue of role of phages in biological dynamics of manure thus formed over years. Firstly, the host bacterium was isolated and identified as *Bacillus cereus* group member as assessed by phylogenetic analysis and secondly it's corresponding phage from same soil sample was also enriched and characterised. The phage (VTCCBPA38) was found to belong to family *Myoviridae* and was active within the temperature range of 4 °C - 45 °C. As assessed by biological sensitivity by spot test, the phage was active against 6/19 (31.6 %) Bacilli tested including *Bacillus cereus* from goat mastitis. Thus the phage may find potential use in biocontrol of diseases caused by *Bacillus* sp. Furthermore, this report is valuable as the first study for investigation of *Bacillus* sp. phage in carcass burial sites.

Keywords: *Bacillus sp.*; Phage; Characterisation; Antibiotic susceptibility; *Myoviridae*; TEM

1. INTRODUCTION

Bacteriophages are recognised as the most abundant biological entities on earth (with an estimated diversity of 10^{30}) and can be found at all sites where bacteria reside[1,2]. Bacteriophages have found application in human therapeutics in few countries such as Georgia; apart they have played a major role in biotechnological developments and now have emerged as an alternative to overcome upcoming antibiotic resistance in pathogenic bacteria. The phages of *Bacillus* sp. - a Gram positive spore forming bacteria, are of particular interest due to the abundance of host in soil and the vast applications of *Bacillus* sp. in biotechnology as well as due to the disease causing potential of *Bacillus anthracis*. *Bacillus cereus* of this group is often referred to as a food contaminant and serves as an opportunistic pathogen in humans. *Bacillus cereus* has been reported as a causative agent of Gangrenous mastitis in goats[3] and has been isolated from mastitic milk[4] also. Though the phages infecting *B. cereus* are diverse in nature but majority are described as lysogens. In the current study, the prevalence of lytic bacteriophage against *Bacillus* sp. isolated from equine carcass disposal site was explored and the host and corresponding phage (both co-existing in the carcass soil) were characterised further to explore their future use in biocontrol.

2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of Bacteria

Soil samples were collected from an equine breeding farm, Hisar, from the site of carcass disposal and were streaked on nutrient agar (NA) plates. The individual bacterial colonies obtained after overnight incubation at 37 °C were picked, purified and preserved at -80 °C in glycerol. The bacterial cultures were Gram stained and morphology assessed at 100X magnification under microscope (Nikon DS-Ri1).

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2.2 Bacterial Characterisation

DNA isolation, antibiotic sensitivity, PCR for 16s rRNA gene and phylogenetic analysis are as follows.

2.2.1 DNA Isolation

Bacterial genomic DNA was isolated using ZR fungal/ Bacterial DNA kit (Zymo Research Corporation, Irvine, CA, USA) from freshly grown cultures. 50 µL nuclease-free water was used to elute the DNA and the quality and concentrations were assessed using Nanodrop spectrophotometer.

2.2.2 Antibiotic Susceptibility of Bacterial Isolates

A sterile cotton swab was used to transfer 16h broth culture of the isolated strain by aseptically dipping the swabs into tubes and streaking on to the Mueller Hinton Agar (MHA) plates. The antibiotic resistance of the bacterial isolates was tested against specified antibiotic discs using the disc diffusion method. Growth of the bacteria on the plates was observed after 16 h incubation at 37 °C.

2.2.3 PCR Amplification of 16s rRNA Gene

Bacterial genomic DNA was used for PCR amplification of 16s rRNA encoding gene[5]; amplicons were cloned into pGEM-T Easy vector and got sequenced commercially.

2.2.4 Phylogenetic Analysis

NCBI database was used to retrieve the gene sequences of all the related members for phylogenetic analysis and these were aligned by using ClustalW method of molecular evolutionary genetic analysis (MEGA5) program[6]. Phylogenetic tree was constructed using Neighbor-Joining method in MEGA5 with bootstrap analysis using 1000 replicate.

2.3 Bacteriophage Enrichment and Isolation

All bacilli were used as a host to enrich bacteriophage from soil samples. For this, one gram of soil was added to PBS and mixed with equal volumes of grown bacterial culture and 5x nutrient broth (NB) and incubated at 37 °C for 8-12 h, afterwards the mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through a 0.22 µm PVDF filter. Spot assay was performed by placing 5 µl of filtrate on nutrient agar (NA) seeded with host bacteria to detect the bacteriophage activity. To obtain plaques, the enrichment host was plated with serially diluted filtrate by using double agar layer technique. One clearly separated plaque was purified three times by picking in SM buffer (5.8 g/L of NaCl, 2.0 g/L of $MgSO_4$, 50 mL/L of 1 M Tris, pH 7.5, % mL/L of presterilised 2 per cent gelatin) and replating. Plaque characteristics were recorded and phage titre was determined.

2.4 Phage Bulk Culture

For bulk culture, 2 mL of exponentially growing host culture was pelleted and suspended in SM buffer. Phage suspension (10^9 PFU) was inoculated into it and incubated at 37 °C for 20 min, after which it was added to NB and incubated at 37 °C for 8-12 h under shaking conditions. Thereafter, it was kept for 10 min after the addition of chloroform without disturbance, followed by centrifugation for 10 min at 8300 rpm. A final concentration of 1M NaCl and 10 per cent PEG800 (Sigma Aldrich) were added to the supernatant. Bacteriophage pellet was dissolved in SM buffer and the pellet was repurified by extraction with chloroform. The isolated bacteriophage preparation was preserved in NCVTC with Accession no. VTCCBPA38.

2.5 Protein Profile

The protein profiling was carried out by SDS-PAGE and staining with coomassie stain[7]. The generated profile was analysed using AlphaEaseFC software (Alpha Innotech).

Figure I. Phylogeny of *Bacillus* sp. on the basis of 16s rDNA sequence.

2.6 Temperature Sensitivity Assessment

For temperature sensitivity assessment, phage suspension was incubated at 4 °C, 25 °C, 37 °C, 45 °C, 55 °C, 65 °C and 80 °C for 1 h before plating and plaques were enumerated.

2.7 Transmission Electron Microscopy

The phage suspension was placed on carbon coated copper grids and negatively stained with 1 per cent phosphotungstic acid, pH 7 to perform the electron microscopy. JEOL (Welwyn Garden City, England) electron microscope operating at 80 Kv was used to take the electron micrographs.

2.8 Biological Activity of Bacteriophages

The biological activity of the bacteriophages against various bacterial isolates was tested. Bacterial lawn was spotted with 2 µl of the concentrated phage suspension and incubated overnight at 37 °C. After incubation, the development of the clear zone was indicative of bactericidal activity.

3. RESULTS

3.1 Bacterial Isolation, Identification and Phylogeny

Bacterial isolate staining Gram positive, filamentous bacilli was isolated from the soil sample. The 16s rRNA amplicons were cloned and sequenced successfully and upon phylogenetic analysis, the bacterium was identified to be *B. cereus* group member as shown in Fig 1.

3.2 Antibiotic Susceptibility Test

Bacteria showed resistance to a number of antibiotics. The results are indicated in Table 1.

Table I. Antibiotic susceptibility testing of *Bacillus cereus* isolate obtained from cadaver affected soil. (S) denotes sensitive and (R) denotes resistant

Antibiotic	Concentration (mcg/disk)	Susceptibility
Amikacin	30	S
Ampicillin	10	R
Aztreonam	30	S
Amoxyclav	30	R
Cefepime	30	R
Cefotaxime	30	R
Cefpodoxime	10	R
Cephalothin	30	R
Ceftazidime	30	R
Ceftizoxime	30	R
Cefuroxime	30	R
Co-Trimoxazole	25	S
Gentamicin	10	S
Imipenem	10	S
Ofloxacin	30	S
Tetracycline	30	S
Tobramycin	10	S

3.3 Bacteriophage Isolation

When the purified host was used to enrich naturally occurring phages from cadaver affected soil, bacteriophage was detected by spot test. The bacteriophage against this bacteria produced plaques with clear, pinpointed, rough or irregular margins and titre in PEG concentrate upto 2.7 x 10^{11} was obtained.

3.4 Protein Profiling of Bacteriophages

The PEG concentrated bacteriophage was used to generate SDS-PAGE profile and it was observed that bacteriopahge -VTCCBPA38 showed major protein band of ~48 kDa and another protein band of ~68 kDa.

3.5 Temperature Sensitivity of Bacteriophages

The bacteriophage isolate was found to be active within the temperature range of 4 °C to 45 °C and lost it's activity upon further raising the temperature.

3.6 Electron Microscopy

Morphologically bacteriophage VTCCBPA38 against *Bacillus cereus* belonged to family *Myoviridae* and had an isometric head of 69 nm diameter and a tail of 131 nm with visible base plate as shown in Fig. 2.

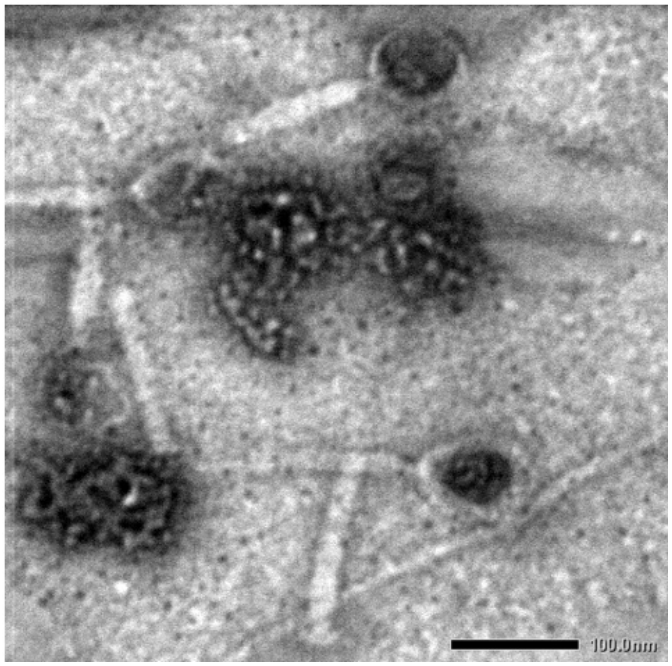

Figure 2. Morphology of bacteriophage VTCCBPA38 against *B. cereus.*

3.7 Biological Activity of Phages

The bacteriophage was tested for it's biological activity against a total of 19 bacterial *Bacillus* spp. isolates and it showed activity against 6/19 (31.6 %) of the tested bacteria as shown in Table 2.

4. DISCUSSIONS

Many a times the equines face threats of epidemics which are controlled by disposing off the infected animals. The onsite burial is the method of choice however the most neglected and

Table 2. Biological activity of phage -VTCCBPA38 against other Bacilli

Bacterial isolate	Details	Activity of VTCCBPA38
VTCCBAA3	*B. subtilis* from soft tick	-
VTCCBAA166	*B. subtilis* from canine wound	-
VTCCBAA180	*B. subtilis* from bovine	-
VTCCBAA182	*B. cereus* from bovine	+++
VTCCBAA183	*B. cereus* from bovine	-
VTCCBAA330	*B. pumilus* from equine intestine	-
VTCCBAA443	*B. cereus* from equine dung	-
VTCCBAA479	*B. mycoides* from bovine vaginal swab	+
VTCCBAA480	*B. mycoides* from bovine lung tissue	+++
VTCCBAA490	Bacillus spp. from equine	+++
VTCCBAA567	*B. cereus* from porcine	-
VTCCBAA692	*B. cereus*	+++
VTCCBAA737	*B. cereus*	-
VTCCBAA790	Bacillus spp. from bovine	-
VTCCBAA792	Bacillus spp. from bovine vaginal discharge	-
VTCCBAA805	*B. licheniformis* from canine	-
VTCCBAA807	*B. cereus* from goat mastitic milk	+++
VTCCBAA915	Bacillus spp. from equine	-
VTCCBAA952	Bacillus spp. from sheep stomach tissue	-

equally important issue always prevails regarding assessment of the spread of bacterial contaminants in the soils of cadaver sites and monitoring the soil microbial flora on regular intervals. In the current study we assessed the *Bacillus* sp. and it's phages prevailing in the soil from cadaver disposal site of an equine production farm. The bacterial isolate was identified by 16s rRNA sequence analysis and was found to resemble *Bacillus cereus* and *B. weihenstephenensis* by phylogenetic analysis (Fig. 1). Though filamentous bacilli are natural inhabitants in soil but current report describes phage against *Bacillus* sp. host bacteria isolated from equine cadaver affected soils and about host and corresponding phage being isolated from the same sample.

The thickly staining *Bacillus* spp. isolate was identified as a member of *Bacillus cereus* group and was resistant to ampicillin, amoxyclav and cephalothin which are generally effective against Gram positive bacteria. The isolate also showed resistance for 3rd and 4th generation antibiotics as indicated in the Table 2. *Bacillus* group - which includes entomopathogens and etiological agents of foodborne illness or anthrax, persists in various environments and various strains have been isolated from diverse habitats of soil[8]. Many keratin degraders derive from the bacterial genus *Bacillus* and two strains of *Bacillus* spp. including one of *Bacillus cereus* group has been found to exhibit keratinolytic property hence it may indicate an implication of the presence of such a microbe from the current site of animal cadaver disposal[9]. A *Bacillus cereus* strain has been proved to exhibit multi traits activity which may be extremely useful for biotechnological processes involving keratin hydrolysis, feather biodegradation or in the leather industry[10]. Resistance against penicillin in *B. cereus* isolates has previously been reported[11-12] as we observed in our isolate as well.

Phages are abundant and temporally dynamic members of the environment. As they infect and replicate in specific host so, the relative abundance of corresponding phage types roughly parallels that of the host. Bacteria existing in an environment depend on the prevailing phages as they keep in check competitively dominant species or populations and hence they tend to change the population dynamics[13]. An immense diversity of phages exist in environment[14]. It has been estimated that the phages outnumber bacteria by an estimated tenfold[2], however only a very small fraction of environmental phages have been properly characterised. Reason being, many host bacteria cannot be still cultivated under laboratory conditions and hence bacteriophage sequences have been amplified using PCR based and more recently via metagenomic approaches. The phage abundance is again substantiated by frequency of novel genes found in newly characterised phage genomes[15]. In the present study, we were able to obtain a bacteriophage producing clear, pinpointed plaques on the host bacteria. The SDS-PAGE profile indicated the presence of ~48kDa protein band which is indicative of *Myoviridae* family. This was confirmed by transmission electron microscopy where a *Myoviridae* phage with isometric head of 69 nm diameter and a tail of 131 nm was clearly visible against the isolated *Bacillus cereus* from the soil of a cadaver disposal and burial site.

The biological activity assessment by spot test indicated that the phage could lyse 6/19 (31.6 %) including *B. cereus* from goat mastitis indicating that the phage may be useful in typing as well as biocontrol of pathogenic and non-pathogenic *B. cereus*.

5. CONCLUSIONS

Current study, elaborates the isolation and characterisation of bacterial host and it's corresponding phage from horse cadaver affected soil. The host bacteria (*B. cereus*) and it's corresponding lytic phage were located together in the soil: it indicates the prevalence of soil dynamics being in harmony where the predator and the prey were present together and this could be a mode of population control. Therefore, this study was necessary to monitor microbial safety of ground soils of burial sites for ensuring health of the people who are associated with farm activities. Furthermore, this report is valuable as the first study for investigation of *Bacillus* sp. phage in carcass burial sites.

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