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Research Article

Isolation, identification, characterization and evaluation of antibacterial activity of bacteriophages against *Salmonella* spp. isolated from cattle, poultry and pigs in North eastern region of India

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Abstract

Antimicrobial resistance is a global concern for human and animal health. Development and commercialization of new antibiotics are difficult, time-consuming and extremely costly; hence, exploration of alternatives is a focused area of research. Bacteriophages, as alternative to antibiotics, are one of the most suitable sources. The present study was undertaken to isolate and identify bacteriophages from sewages and to evaluate their lytic activity against MDR *Salmonellae* isolated from domestic animals in Mizoram. A total of 50 samples were processed for isolation, identification and characterization of bacteriophages. Similarly, 197 rectal swabs from cattle, pigs, and poultry were processed for isolation and identification of *Salmonella*. All the isolates were subjected to determination of their antimicrobial sensitivity profile by disk diffusion technique. All the bacteriophage isolates were assessed for their lytic effect against the MDR *Salmonella* isolates. Total six bacteriophages were isolated from sewage samples. Transmission electron microscopy revealed that bacteriophages had icosahedral capsid head, necks, and contractile tails with tail fibres, therefore, belong to *Myoviridae* family. Total 28 *Salmonella* were isolated. By disk diffusion assay, majority of the *Salmonella* isolates exhibited resistance against commonly used antibacterial agents, *viz*., amoxicillin (92.31%), ceftriaxone (88.46%), tetracycline (80.77%), doxycycline (76.92%), while imipenem and meropenem exhibited 100% sensitivity. Four bacteriophages exhibited lytic activity against MDR *Salmonella* isolates. Bacteriophages isolated and identified from sewages of Mizoram exhibited antimicrobial activities against field isolates of multidrug-resistant *Salmonella*. Bacteriophages can be considered as a potential alternative to antibiotics to control of the menace of AMR.

Keywords: Antibacterial, Bacteriophages, Mizoram, *Salmonella*

Highlights

- *Salmonella* isolated and identified from cattle, pig, and poultry in Mizoram
- Bacteriophages are isolated and identified from sewages in Mizoram
- Bacteriophages exhibited lytic activities against MDR *Salmonella* isolated from animals

INTRODUCTION

Antimicrobial resistance (AMR) is a global health concern and due to its ever-increasing phenomena the World Health Organization has issued the warning of the dangers of a post-antibiotic age, in which common diseases might re-emerge as lethal diseases. In a report published in 2019, bacterial infection killed an estimated 4.95 million people (3.62-6.57), with 1.27 million deaths were directly attributable to bacterial AMR (Antimicrobial Resistance Collaborators, 2022). According to reports, the beta-lactams, polymyxins, tetracyclines, and macrolides, which are mostly used to treat intestinal and respiratory infection in human and animals are the most often used in animal production also as prophylactic agent or growth promoter (Burow et al., 2019).

Salmonella, one of the most common food-borne pathogens worldwide, poses a multifaceted threat to food production and safety, which is exacerbated by the emergence of antimicrobial-resistant strains. According to the current EFSA/ECDC study on zoonoses and zoonotic diseases, pork and associated products were responsible for 9.3% food-borne incidents caused by *Salmonella* in Europe (European Food Safety Authority & European Centre for Disease Prevention and Control [EFSA & ECDC], 2015). Similarly, a "farm-to-fork" model used in the United States predicted that pork was responsible for about 100,000 cases of human salmonellosis each year (Miller et al., 2005). Intestinal commensal bacteria in both animals and humans are considered good indicators of the general level of antimicrobial

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resistance as they are exposed to selection pressure driven by any antimicrobial treatment of their host (Blake et al., 2003; Szmolka & Nagy, 2013). Resistance can be passed from commensal (e.g., *E. coli*) to pathogenic (e.g., *Salmonella* spp.) bacteria (Blake et al., 2003). In addition, the biofilm producing multidrug resistant (MDR) pathogenic bacteria are considered as a major concern to public health. Majority of the antimicrobial drugs available in the arsenal of the medical and veterinary practitioners are becoming gradually ineffective due to the development of resistance (Dutta, 2020). The new generation antimicrobials are costly and limited for routine use for the common man in the middle-and-low-income group of countries (Ghosh et al., 2019). With the huge threat posed by the MDR bacterial pathogens there is need to develop safe, dependable and cost-effective alternatives to counter the menace of ever-increasing threat of AMR. The research and introduction of new antibiotics into doctors' arsenals are unable to keep up with the pace at which bacteria develop resistance, which increases the interest in alternative microbial control technologies to grow (Plackett, 2020). One of the most powerful and successful approaches to combat multi-drug resistant (MDR) bacteria is bacteriophage and the products derived from it (Czaplewski et al., 2016).

Bacteriophages are widely distributed in nature and have a life cycle that is strictly associated with the bacterial cells (Zhan et al., 2015). The antibacterial action of phages was discovered for the first time separately by Hankin, Gamaleya, and Twort in 1896, 1898, and 1915, respectively (Sulakvelidze et al., 2001). Soil, sewage, and animal secretions are the most common sources of phages, allowing them to be isolated for therapeutic purposes (Simmonds & Aiewsakun, 2018). Phage therapeutic strategies may be one of the most extensively used therapies for infections brought on by antibiotic-resistant bacteria in the current period of limited antibiotic supply (Kutter et al., 2015; Domingo-Calap et al., 2016; Gorski et al., 2018).

There is very limited information available on isolation, identification and antimicrobial activity of bacteriophages from India and no data is available from Mizoram. Accordingly, the present study was conducted on isolation, identification and molecular characterization of bacteriophages from sewage to evaluate their antimicrobial property against *Salmonella* spp. isolated from pigs in Mizoram, India.

MATERIALS AND METHODS

Collection of samples: Sewage samples (n=50) were collected in sterile containers from various places of Mizoram following the method described by Jothikumar et al. (2000) and carried to the laboratory under cold chain for further processing.

Isolation and identification of bacteriophages: All the samples were inoculated in 5 mL nutrient broth (10X) along with 1 mL of overnight incubated *E. coli* (ATCC 43888) in LB broth, 400 µL of 0.1M calcium chloride and 0.1M magnesium chloride. The entire mixture was incubated aerobically at 37°C overnight in a shaking incubator at 150-200 rpm. Following incubation, the enriched broth was centrifuged at 10000 rpm for 15 minutes followed by collection of supernatant and sequential filtration through 0.45 µm and 0.2 µm membrane filters. The filtrate was stored at 4°C for further use. As described by Adams (1959), double agar overlay method was performed to isolate the bacteriophage using hard agar at the bottom as the base and a mixture of phage particles and host cells (*Escherichia coli* ATCC 43888) in soft agar at the top to demonstrate plaques following incubation.

The phage isolates were characterized by turbidity reduction test in *E. coli* (ATCC 43888) as per the method described by Shende et al. (2017) and spot assay as described by Adams (1959).

Estimation of phage titre: Estimation of phage titre was done by enumerating the visible plaques in double agar overlay plate. Plaque-forming unit (PFU) could be used to measure the viable bacteriophage concentration using following equation (Pelzek et al., 2013).

$$\text{PFU/mL} = (\textit{No. of visibleplaques}) \times \frac{1000}{\mu\textit{lofphagestockplate}} \ (\textit{dlutionfactor})$$

Molecular characterization of bacteriophages by restriction fragment length polymorphism: The phage isolates were concentrated with 1M NaCl and PEG 8000 (10% wt/vol) and was kept overnight at 4°C to obtain the bacteriophage pellet, which was further dissolved in phage buffer (Anand et al., 2017). Phage DNA was extracted as per the method described by Jothikumar et al. (2000). Extracted DNA were checked for their purity and quantity by Nano-drop spectrophotometer and stored at -80°C for further use.

Restriction fragment length polymorphism (RFLP) was performed as per the method described by

Goodridge et al. (2003) for identification of bacteriophage isolate. As mentioned in Table-1, phage DNA were digested with *Eco*RI, *Bam*H1, *Xho*I and *Hind*III restriction endonucleases following the guidelines of the manufacturers and incubated for 4 hours at 37°C. The digested DNA fragments were separated by electrophoresis in 1% agarose gels containing ethidium bromide (0.5 µg/mL) in TAE buffer at 30 V for 6-8 hours. The fragments were visualized in a UV transilluminator and documented using gel documentation system. Fragment sizes were determined from co-migrating *Eco*R1 and *Hind*III digests of lambda DNA.

Morphological documentation bacteriophage isolates by Transmission Electron Microscopy (TEM): Transmission electron microscopy was used to examine the morphology of the bacteriophage isolates applying 60 keV accelerating voltage as described by Goodridge et al. (2003). To observe the phage morphology, 100 µL of the phage suspension (1011 PFU/mL) was placed on the carbon coated copper grid (200 mesh) and the sample was allowed to stay until get dried followed by staining with 1% uranyl acetate and allowed to dry. After drying, the samples were examined in a FEI TECHNAI G2 F20 X-Twin, transmission electron microscope to observe and document the phage morphology.

Isolation, identification and characterization of *Salmonella* spp.: A total of 197 rectal/cloacal swabs (Cattle = 81, pigs = 40 and poultry = 76) were collected from different places of Mizoram and processed for isolation and identification of *Salmonella* spp. following the standard bacteriological techniques as described by Edwards and Ewing (1972). All the isolates were characterized phenotypically using their cultural, morphological and biochemical properties as described by Quinn et al. (2004). All the tentatively suspected isolates were further confirmed by VITEK 2 automated bacterial identification system.

Phenotypic determination of antibiotic sensitivity profile of *Salmonella* isolates: All the *Salmonella* isolates under the study were subjected to AST by disk diffusion assay on Mueller-Hinton agar (HiMedia, Mumbai) plate as per the guidelines of Clinical and Laboratory Standards Institute [CLSI] (2020) using 23 commercially available antimicrobial disks.

Determination of antibacterial property of the bacteriophage isolates: The antibacterial property of bacteriophages was conducted against the *Salmonella* isolates as per the protocol described by Kutter and Sulakvelidze (2005). In brief, sterile LB agar was poured in plates and let to solidify, followed by overnight incubation at 37°C for sterility check. Then 0.7% sterile LB soft agar along with 0.1M of calcium chloride, 0.1M magnesium chloride, 100 µL of reference strain of *E. coli* (ATCC 43888) and the field isolates of *Salmonella*, which were grown for 4-6 hrs at 37°C was mixed and transferred directly onto LB agar plates.

The titre of the phage was determined by plaque assay as per method given by Pelzek et al. (2013) and different concentrations of the phage suspension (10^8 PFU/mL) was put on the bacterial layer on LB plate and incubated at 37°C. The plates were checked after 4–6 hrs and again after 18 hrs for visualization of bacterial lysis.

RESULTS

Isolation of bacteriophages: In the present study, based on the turbidity reduction test, spot assay, double agar overlay assays, plaque assay, TEM and RE analysis a total of 6 bacteriophages (P1 to P6) were isolated and identified from the sewage samples collected from various locations of Mizoram.

In the present study, the plaque assay for confirmation of bacteriophages in the specimens revealed small or large, clear or opaque and diffused plaques with a diameter of 0.1 to 3 mm (Fig. 1). The PFU was calculated based on number of countable plaques in one mL of specimens by serial dilution method. Detail result of the plaque assay is depicted in Table- 1, 2, and 3.

In the present study, TEM observation of the phage virion revealed the icosahedral capsid head and a contractile tail, which is characteristic of *Myoviridae* family.

Table 1. Details of plaque assay for demonstration of bacteriophages by double agar overlay method

Plaque morphology		No. of isolates
Size	**Density**	
Small sized	Clear	2
Small sized	Opaque	1
Large sized	Clear	1
Diffused	Clear	1
Diffused	Opaque	1
Total		6

Molecular characterization of bacteriophages: Phages appear to have dissimilar profiles of the nucleic

Table 2. Determination of plaque-forming units (PFU) of the bacteriophage isolated from sewages of Aizawl, Mizoram

SL. No.	Sample ID	No. of visible plaques at different dilution							
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1.	P1	Uncountable			≥300	≥200	42		
2.	P2	Uncountable			≥300	≥200	12		
3.	P3	Uncountable			≥200	≥150	18	No visible	
4.	P4	Uncountable				≥200	23	Plaques	
5.	P5	Uncountable				≥200	10		
6.	P6	Uncountable			≥200	≥150	27		

Table 3. Enumeration of plaque-forming units (PFU/mL) of the bacteriophage isolated from sewages of Aizawl, Mizoram

SL. No.	Sample	Dilution Factor	No. of visible plaques	µL of phage plated	Plaque forming units (PFU/mL)
1.	P1	10^{-6}	42	100	$4.2x10^{-8}$
2.	P2	10^{-6}	12	100	$1.2x10^{-8}$
3.	P3	10^{-6}	18	100	$1.8x10^{-8}$
4.	P4	10^{-6}	23	100	$2.3x10^{-8}$
5.	P5	10^{-6}	10	100	$1x\ 10^{-8}$
6.	P6	10^{-6}	27	100	$2.7x10^{-8}$

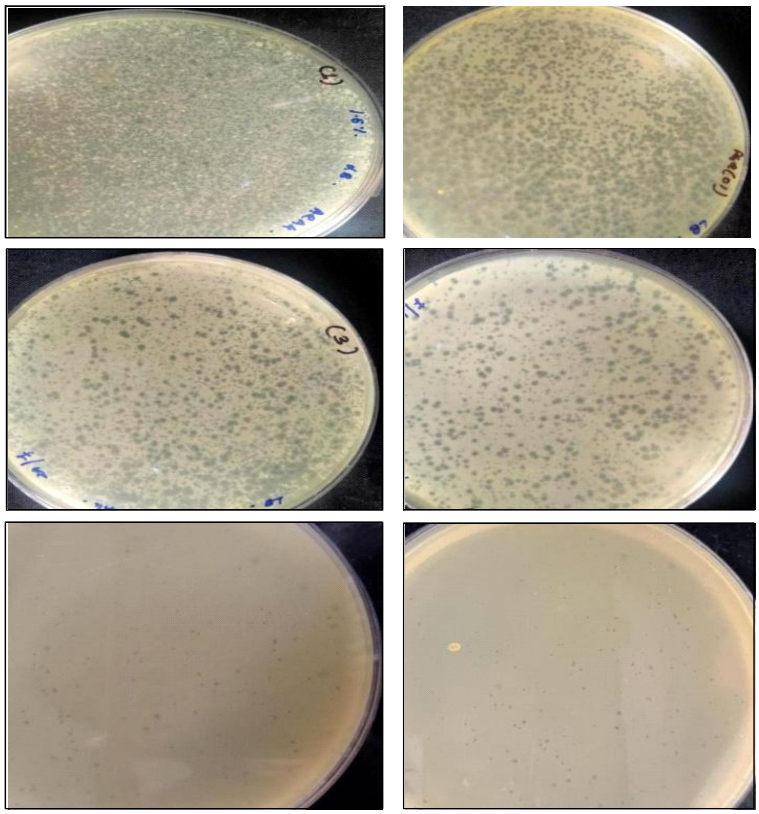

Fig. 1. Plaque assay for demonstration of bacteriophages isolated from different sewage samples collected from various places of Aizawl

acid fragments generated by digestion of their DNA with restriction enzymes like *Xho1*, *Eco*RI, *Bam*H1 and *Hind*III. The nucleic acid under restriction endonuclease analysis revealed the presence of four restriction sites with *Hind*III enzyme, where the nucleic acids have been digested partially by *Eco*R1. The restriction enzyme digestion revealed that phages have DNA as their genetic material (data not shown).

Isolation and identification of *Salmonella*: A total of 28 *Salmonella* were isolated and identified. All the *Salmonella* isolates were grown as small and pale colonies on MacConkey's agar, followed by typical black centered colonies on xylose deoxycholate agar (XLD) medium. All the isolates were positive for methyl red tests and citrate utilization test and negative for indole, Voges-Proskauer and oxidase test and negative for urease test. All the isolates fermented glucose, maltose, and mannitol.

Antimicrobial sensitivity profile of *Salmonella*: In the present study, amoxicillin (92.31%) exhibited highest level of resistance against all *Salmonella* isolates, followed by ceftriaxone (88.46%), tetracycline (80.77%), doxycycline (76.92%), while imipenem and meropenem exhibited 100% sensitivity.

Antibacterial property of bacteriophages against *Salmonella*: All the 6 bacteriophage isolates (P1 to P6) were tested for their antibacterial property against all the field isolates of *Salmonella* (n=28), of which P1 (3.85%), P2 (7.69%),

P4 (3.85%), and P5 (7.69%) exhibited lytic activity. The P3 and P6 isolates did not show any lytic activity against any of the isolates.

DISCUSSION

In the present study, the recovery rate of *Salmonella* spp. was 14.9%, 15% and 19.74% from cattle, pigs and chicken of Mizoram. Earlier, from the same laboratory, Kylla et al. (2019) isolated 38 (8.31%) *Salmonella* spp. from 457 fecal samples collected from pigs of Manipur, Meghalaya, Mizoram, and Nagaland. Murugkar et al. (2005) reported a rate of 20.5% *Salmonella* from humans, poultry, and animals, including pigs in North Eastern India. Various workers from other countries also reported isolation rate of *Salmonella* spp. ranging between 5.22%-9% from different animal sources (Akortha & Filgona, 2009). Basavaraj et al. (2011) recovered 1.8 % *Salmonella* spp. from North Karnataka, India, when surveyed the tertiary care hospital patients. Nagshetty et al. (2010) isolated a total of 95 *S.* Typhi isolates from 1250 samples, which revealed an incidence rate of 7.6%. However, Shekhar and Singh (2015) from Uttarakhand could isolate only 14 *Salmonella* isolates (2.05%) from 682 samples. Prevalence of *Salmonella* spp., and other enteric organisms in human or animals is always variable depending upon the geographical location, nutritional factor as well as immune status of the individuals, from where the samples are obtained. Presence of comparatively high rate of *E. coli* in gut microflora of human or animal is a common phenomenon because they are considered to be a commensal organism. However, isolation of *Salmonella* spp., from the faeces of human or animals at any level is an indicator of pathological conditions. All the isolates obtained in this study revealed typical cultural and biochemical characteristics of *Salmonella* as mentioned by Ewing (1986).

Majority of the *Salmonella* isolates under the present study were found to be highly resistant to amoxicillin (92.31%), followed by ceftriaxone (88.46%), tetracycline (80.77%), nalidixic acid (76.92%), and doxycycline (76.92%). Imipenem and meropenem were found to be the most sensitive (100%) antimicrobial agents against all the salmonellae. Bergeron et al. (2010) reported antibiotic susceptibility test of 33 *S.* Typhimurium isolated from swine, which revealed the most prevalent resistance profile against ampicillin and tetracycline. Habrun et al. (2010) reported the highest sensitivity rate of 42 *Salmonella* isolates in Croatia to colistin and enrofloxacin (100% both), whereas 76% of isolates showed resistant to ampicillin and ampicillin/clavulanic acid. Das and Manefield (2012) isolated 16 isolates of *Salmonella* Typhi from humans. All the 16 (100%) isolates were found resistant to ampicillin, moderately sensitive to nalidixic acid and nitrofurantoin and sensitive to carbenicillin, chloramphenicol, and gentamycin. Nagshetty et al. (2010) reported antibiotic susceptibility test of 95 *S.* Typhi, which revealed that all the isolates of *S.* Typhi were sensitive to imipenem. Highest resistance was observed against nalidixic acid (31.57%), closely followed by ampicillin (29.47%) and chloramphenicol (28.42%). Resistance against ciprofloxacin and ceftriaxone was only 4.21% and 6.31%, respectively. This was in corroboration with the present finding in which ciprofloxacin and ceftriaxone were found to be amongst the least resistance antibiotics tested.

In the present study, total 6 bacteriophages (P1 to P6) were isolated from sewages of different places of Aizawl, Mizoram. Based on a study of past literatures, we attempted to investigate different sources such as soil and pig sty effluents. But bacteriophages could not be recovered from soil and pig effluents. Based on their location, season, and the intended sampling site, the isolation rate of bacteriophages from sewage or other sources remained variable. Bacteriophages are obligatory parasite and require bacterial host for their sustainability, either in their lytic and/or lysogenic cycle (Shende et al., 2017). Phages were more frequently isolated during the summer season before monsoon starts (Comeau et al*.,* 2005) might be due to high concentration of bacterial substrate in the sewages or other effluents. During rainy seasons, the bacterial load decreases in the soil of effluents, which also reduces the rate of propagation of bacteriophages. In our present study, all the bacteriophages were isolated during summer months when rain fall was very less and no phages could be recovered after that, which is in corroboration with the previous reports (Shende et al., 2017).

In the present study, TEM observation of the phage virion revealed the icosahedral capsid head and a contractile tail, which is characteristic of *Myoviridae* family. Karpe et al. (2016) examined the presence of bacteriophages by TEM, which belonged to order *Caudovirales* and based upon their icosahedral head and contractile tails, phages belong to the *Siphoviridae*, *Myoviridae* family. Previous reports shown that tailed phages (*Caudovirales*) are the most diverse, numerous, and widely distributed of all bacterial viruses (Aprea et al*.,* 2015). Interestingly, the current study found that the most common lytic phages

infecting *E. coli* and *Salmonella* belonged to the *Myoviridae*, followed by *Siphoviridae* and *Podoviridae*, which is consistent with the prior reports (Karpe et al., 2016; Kumar et al, 2022). The nucleic acid under restriction endonuclease analysis revealed the presence of four restriction sites with *Hind*III enzyme, where the nucleic acids have been digested partially by *Eco*R1. The restriction enzyme digestion revealed that phages have DNA as their genetic material, which is consistent with the report published elsewhere (Bao et al., 2011; Bardina et al., 2012). *Bam*HI could not digest the DNA of any of the phage isolate, which might be due to the non-availability of any restriction site in the phage genome or due to recent mutation in their genome.

In this investigation, we isolated bacteriophages from all samples using only one bacterial host (*E. coli*, ATCC 43888). In previous reports, various workers reported *Bacillus cereus* and *Bacillus subtilis* as the best host for growth of bacteriophages (Shukla et al., 2014; Shende et al., 2017). There are reports indicating the use of either *B. subtilis* and/or *E. coli* (Yuan et al., 2012; Gillis & Mahillon, 2014). *B. subtilis* supported the growth of multiple morphotypes of phages as compared to *E. coli* (Shende et al., 2017). Similarly, Krasowska et al. (2015) also reported that phage had the highest per cent adsorption to the *Bacillus* host. Due to high activity at the low and high temperature and pH, *B. subtilis* phage seems to be the best candidate for use in isolation work. Less recovery of *E. coli* phages may be correlated with the size of bacterium (Shukla & Hirpurkar, 2011). *E. coli* being smaller in size provides less surface area for attachment of bacteriophage as compared to *B. subtilis*. In the present study, we have used only one specific host keeping in mind that the bacteriophage isolates will be tested for their antibacterial activities against members of family *Enterobacteriaceae* only.

To investigate bacteriophage's ability to lyse *Salmonella* spp. *in vitro*, the double agar overlay method was performed, which include the addition of phage ranging from 10^6 to 10^8 PFU/mL. Bacteriophages are considered to be a powerful antibacterial agent because they are advantageous, that is, readily available, naturally occurring, specific in their activity, and capability of rapid multiplication in presence of their host (Gill & Hyman, 2010). It also emerged as new antimicrobial agents in recent decades, with significant activity in monitoring environmental quality, biofilm control, and water treatment (Raghu et al., 2012). In the present study, the lytic activity of bacteriophages was tested against 28 *Salmonella* isolates, of which 14.29% showed the lytic activity. The lower lytic efficacy of bacteriophage against the clinical isolates of salmonellae might be due to the alteration of receptors, leading to the decrease in binding affinity. In an earlier report it is accepted that bacteriophages from sewage were able to infect broad host range and not highly serovar specific (Carey-Smith et al., 2006). On the other hand, phages in water sewage can reach a wide variety of bacterial hosts (e.g., different *Salmonella* serovars). The virulent phages, st104a, st104b and Felix 01 isolated from water sewages were also reported to be infective over a wider range of Salmonellae serovar hosts (O'Flynn et al., 2006). In another study, 34 *Salmonella* phages isolated from sewage samples and determined host range by spot testing against 53 different serovars revealed that most of those phages were only effective in lysing the host strains, but only 5 phages had a wider range and none of them could infect all tested strains (Heringa et al., 2010). Callaway et al. (2010) reported that lytic phages against *S.* Typhimurium, which had a high degree of specificity. Mohamed et al. (2020) from Egypt identified bacteriophage ZCSE2 from raw sewage with substantial antibacterial activity against multiple multidrug-resistant (MDR) virulent *Salmonella* strains, including *S.* Typhimurium DT104 and *S.* Typhimurium U288. Lu and Koeris (2011) also discovered a phage SS3easa broad host range, which is rare, as most phage host ranges are narrow and often species-specific, posing a significant barrier to phage therapy.

High specificity of the bacteriophages is their greatest advantage, but it is also one of the barriers to their practical application. The high specificity of bacteriophages ensures precise targeting. However, when many different species and intraspecies variants require treatment, such as in the case of a typical bacterial infection, the system must be flexible enough to target a large proportion of these variants. As a result, appropriately broadening and changing the phage's host range is an important step toward realising the application. Although it has been reported that engineering methods can change the host spectrum of phages, this is limited to a few phages that clarify the mechanism of receptor binding protein adsorption on the host, which is still a long way from practical application (Yoichi et al., 2005; Ando et al., 2015). Bacteriophage replication is essentially host genus specific (Ackermann & Wegrzyn, 2014). However, members of family *Enterobacteriaceae* are so closely related that polyvalent phages are common, particularly in the *E. coli-Shigella-Klebsiella* group (Ackermann, 2007). There are only a few phages that

have broad host specificity across bacterial genera (Sillankorva et al., 2011; Choi et al., 2017). Accordingly, the observation in the present study might be considered as one of the specific bacteriophages recovered from sewages. Further studies will be required to characterize the phage isolates and to determine their host range before recommending them as potential antibacterial agent against major MDR bacterial pathogens.

Therefore, it may be concluded that the bacteriophages isolated and identified from sewages of Mizoram exhibited antimicrobial activities against field isolates of multidrug-resistant *Salmonella*. Bacteriophages could be potential candidate in future as alternative to the conventional antimicrobial agents to contain the menace of AMR in the environment.

Conflict of interest: The authors certify that there is no conflict of interest in publication of the article.

Author's contribution: TMS: Laboratory activities, sample collection; TKD: Conceptualization, manuscript preparation; PR: Data analysis; PKS: Manuscript editing; IS: Manuscript editing; SB: Manuscript editing.

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