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ORIGINAL ARTICLE

Antibiotic susceptibility of *Staphylococcus aureus* isolated from subclinical bovine mastitis cases and in vitro efficacy of bacteriophage

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Abstract

Staphylococcus aureus is an opportunistic pathogen that may cause severe infections in livestock, and represents the major cause of mastitis in dairy cows. Currently, instead of using antibiotics, new strategies are sought to reduce this clinical health problem. The aim of this study was to determine the efficacy of phage therapy to kill *S. aureus* strains obtained from farms located at the State of Guanajuato, México. Thirty-six *S. aureus* strains from cow milk with subclinical mastitis were isolated and identified, and the susceptibility to antibiotics and four phages also isolated in this work was tested. It was found that more of 90% of *S. aureus* isolates were not susceptible to six or more antibiotics, and 100% were resistant to penicillin, dicloxacillin, cefotaxime, ampicillin and cephalothin, and 81 and 77%, to tetracycline and cefuroxime, respectively. Fortunately, 100% of *S. aureus* isolates were susceptible to phages used in this work, which was detected as clear zones using specific phage. It was shown for the first time, that phages used in this study are active against pathogenic *S. aureus* and might be incorporated into the therapy as an important tool for the control of staphylococcal bovine mastitis, specially to antibiotic-resistant *S. aureus* strains isolated in farm located at the state of Guanajuato, México; and its use might be extended to other regions inside or outside the country.

Keywords Bovine mastitis · Bacteriophage · *Staphylococcus aureus* · Multi-resistance pattern · Spot test

Introduction

Mastitis is the inflammation of the mammary gland caused by pathogen agents. This disease is one of the largest production concerns in the dairy industry worldwide (Halasa et al. 2007). Mastitis can be caused by a plethora of pathogenic microorganisms, where at least 137 agents are known to cause the disease, such as *Streptococcus agalactiae, Escherichia coli,*

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among others. However, staphylococci remain the main etiological factor of bovine mastitis in many dairy herds (Sender et al. 2017). *Staphylococcus aureus* is considered an agent responsible for contagious intramammary infection (IMI) in cattle, which is of major interest due to the low cure rate of *S. aureus* infections by antibiotic treatment and its ability to persist in a herd in the form of undetected, subclinical infections (Sears and McCarthy 2003). Currently, the most common method of treatment of bovine mastitis is the administration of antibiotics. However, this kind of strategy has shown some disadvantages including: low cure rate, increasing occurrence of resistance, and the presence of antibiotics residues in the milk (Gomes and Henriques 2016). Another problem of humanity faces is the rapid increase of antibiotic resistance seen amongst pathogenic microorganisms (Michael et al. 2014), which is increased by the marked reduction in the development of novel antibiotics (Norrby et al. 2005).

The necessity of identifying novel methods to combat infections caused by antibiotic resistant bacteria is increasing each year. In this sense, bacteriophage represent an excellent alternative to attack multi-resistant bacteria. Bacteriophages (phages) are viruses able to specifically infect and kill the host bacterial species (Haq et al. 2012). They coevolve with their hosts optimizing its spread and release mechanisms from the bacterial cell to the environment and cause (in the case of lytic bacteriophages) lysis of the bacteria. They are also a major driving force in *S. aureus* evolution as a pathogen since many virulence genes are mobilized between different strains by means of transduction (Xia and Wolz 2014). In this work, the objective was to determine for the first time, the efficacy of phage therapy to kill *S. aureus* strains obtained from farms located at the State of Guanajuato, México. This study demonstrated that 100% of antibiotic resistant *S. aureus* isolates were susceptible to phages used in this work.

Material and methods

Study area and herds

The study was carried out in three municipalities from the State of Guanajuato, Mexico: Apaseo El Grande, Huanimaro and Silao, located in the central region of Mexico. Geographically, there are three climatic zones defined in Guanajuato with a pleasant climate with temperatures ranging from 11.7 to 24.2 °C, an average altitude of 2015 m above sea level, and annual average rainfall of 635 mm. Guanajuato is located at west longitude 99°40′–102°6′ and north latitude 21°51′–19°55′. Three small commercial dairy herds were included in this study, which were selected for convenience based on the readiness to participate in the research and the existence of productive and reproductive data at the sampling time. The livestock facilities, nutritional system and practical management of cows, including milking, was similar among of them, and were classified as intensive production dairy herds, according to Villamar and Olivera (2005). Most herds were Holstein-Friesian breed type with different herd sizes.

Animals and milk sampling

Foremilk samples were collected for California Mastitis Test (CMT) scoring, evaluation of the milk appearance in all lactation cows, including a total of 454 animals, following the method described by Schalm and Noorlander (1957). To determine local signs of mastitis, milk color and consistency were assessed as normal or changed (flakes, clots and discoloration).

Somatic cell count and bacteriological analysis

The somatic cell count (SCC) of the milk was measured using a DeLaval cell counter DCC™ (DeLaval, Sweden) as described by Kawai et al. (2013). Once quarter by subclinical mastitis has been identified, teat was disinfected with swabs soaked in 70% (*v*/v) ethyl alcohol. Then, 10–15 mL milk samples were collected in sterile caped tubes and numbered, according to standard procedures by the National Mastitis Council (Hogan et al. 1999). Samples were immediately transported to the Microbiology laboratory under cold chain.

Sixty milk samples from udder quarters affected by SCM were sent to microbiological analysis. Milk samples were serially diluted (10^{-1} to 10^{-3}) with PBS buffer (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl and 1.8 mM KH_2PO_4, pH 7.2). For isolation of *S. aureus*, diluted samples were inoculated in Staph 110 (Merck™, Darmstadt, Germany) and incubated at 37 °C for 72 h in aerobic conditions. Culture plates that showed three or more different colonies, were discarded and registered as contaminated sample (Hogan et al. 1999). Preliminary selection of bacterial strains was carried out using colony morphology, color, and Gram stain; after that, isolates were grown in Luria-Bertani (LB) broth at 37 °C for 72 h, and stocks were prepared in LB broth with 20% (*v*/v) glycerol. Bacterial stocks were stored at −80 °C and used for further experiments.

Microbial molecular typing

Extraction of genomic DNA was carried out by picking one colony from fresh culture plate by using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). The 16S rDNA was amplified by colony-PCR using 10 pM of the universal oligonucleotide set that amplifies both bacterial domains: forward UBF 5'-AGAGTTTGATCCTG GCTGAG-3′ and reverse 1492 R5′-GGTTACCTTGTTAC GACTT-3′. For the amplification of 16S rDNA a proof fidelity enzyme (BioRad) was used under the following conditions:

5 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1: 30 min at 72 °C; and finally, 5 min at 72 °C. An aliquot of 5 μL of amplicons was subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide to visualize the amplified products.

Antibiotic susceptibility testing

Isolates were suspended in 5 mL LB broth at 37 °C to achieve an absorbance at 600 nm of ~ a 0.5 based on McFarland standard. Bacterial cultures were distributed uniformly on Mueller-Hinton agar (Difco, BD, Sparks, MD) by using sterile swabs, and susceptibilities to an assortment of antibiotics were determined by standard disk-diffusion assays for Gram-positive bacteria (Bio-Rad, Hercules, CA, USA). Zones of inhibition (in mm) were recorded after ~18 h of incubation at 35–37 °C. The zones of inhibition were determined and compared with the standards of performance of the supplier to determine whether the tested strain was sensitive (S), intermediate (I), or resistant (R). Twelve antibiotics were used to test Gram-positive bacteria: Penicillin (PE), dicloxacillin (DC), pefloxacin (PEF), cefuroxime (CXM), gentamicin (GE), cefotaxime (CTX); sulfamethoxazole-trimethoprim (SXT), tetracycline (TE), ampicillin (AM), erythromycin (E), ceftazidime (CAZ), and cephalothin (CF).

Bacteriophage isolation and propagation

The isolation of bacteriophage was performed as described previously by Kutter and Sulakvelidze (Kutter and Sulakvelidze 2005) using apathogenic *S. aureus strain*. Two milk samples and udder skin scraping sterile cotton swab from diagnosed cows with clinical mastitis were obtained and centrifuged in a bench-top centrifuge at 10,000 g for 10 min to 4 °C. The supernatant was filter-sterilized (0.22 μm pore diameter) and stored at 4 °C. The filtered supernatant was added in the 5 mL of LB broth with *S. aureus* apathogenic strain exponential middle phase and the samples were incubated to 37 °C to 120 rpm. After 18 h, samples were centrifugated to 3000 rpm for 10 min to eliminated residues of bacterial. Five chloroform drops were added to the supernatant and maintained at 4 °C.

To confirm the presence of phages and evaluated their lytic mechanism, the Spot Test method was carried out as described by Wommack et al. (2009). In brief, 100 μL of *S. aureus* apathogenic strain broth in the final exponential phase was subjected to plaque by extension in the LB solid agar. Samples were incubated at 37 °C for 24 h, and the lytic activities were registered by mean display of clear zone of inhibition. Four isolated strains of bacteriophages were obtained, and their identification was done. Phages with the widest inhibition zone (lytic activity) were selected to perform in vitro assays.

Bacteriophage susceptibility analysis

The phage range activity was determined by the the spot test method (Armon and Kott 1993) against the tweeny-seven different isolates of *S. aureus* obtained in this work. The experimental groups were performed according to the three-different grade of purification of *S. aureus* phage lysate solutions in order to obtain the best purification procedure. Process 1, low purification level (lysate 1); process 2, medium purification level (lysate 2); process 3, high purification level (lysate 3). The experimental groups were performed using the three-different grade of purification of *S. aureus* phage lysate solutions and according to the factors that influence the phage efficiency in the udder: excipient (physiological saline solution, PBS and propylene glycol), phage concentration (0.3, 0.5 and 0.8 mL) and pH (5, 7 and 8). Soft agar (3–5 mL) with 100 μL of an overnight bacterial culture and equal volume of $CaCl_2$ (300 mM) were gently vortexed and spread on the surface of hard agar. Single drops of each phage lysate were spotted on the inoculated hard agar plates, and plates were incubated overnight at 30 °C (Wommack et al. 2009). Bacterial sensitivity was judged visually by spot clarity; clear (+) or no reaction (−). Assays were repeated in triplicate.

Statistical analysis

Data were analyzed with and analysis of variance (ANOVA) with a completely random factorial design. The model included four factors: lysate (three purified level phage), vehicle (three levels: saline solution, PBS and propylene glycol), phage concentration (three levels: 0.3, 0.5 and 0.8 mL) and pH (three levels: 5, 7 and 8); test was carried out in triplicated. Hence, the database was integrated for a total of 243 data (3 lysates × 3 excipient × 3 concentrations × 3 pH's × 3 replications). In this model the first order interactions were evaluated, and the means comparison was done with the Tukey method ($P < 0.05$). Data were analyzed with the Statgraphics Centurion Program v.16.1.

Results and discussion

Bacterial isolates

Using preliminary bacterial identification protocols, such as colony morphology, color, and Gram stain, it was obtained thirty-six putative *S. aureus* isolates (60%). Based on 16 s rDNA sequences, it was confirmed that isolates were *S. aureus.* These strains were catalase positive, and displayed response of mannitol sensitivity and β-hemolysis tests (24%).

Similar result was mentioned by Salgado-Ruiz et al. (2015), which carry out a study in small farms in the highlands

of Central Mexico and they found between 52 to 66% of milk samples were positive for *S. aureus*. Several reports have shown that this bacterium is an important pathogen responsible for contagious intramammary infections (IMI) in cattle worldwide (Sears and McCarthy 2003), and remains as a microorganism of central interest for the dairy industry and veterinary medicine in the herds.

Antibiotic susceptibility patterns

All tested isolates exhibited a perceptible degree of resistance to antibiotics. Tweenty four *S. aureus* isolated of this study (66.6%) showed resistance from six to nine antimicrobial agents, mostly to penicillin, dicloxacillin, cefotaxime, ampicillin and cephalothin while resistance to ten or more antimicrobial agents was found in 12 isolates (33.3%). Only some *S. aureus* strains showed a low susceptibility (44–58%) to gentamycin, pefloxacin, sulfamethoxazole-trimethoprim and erythromycin. Special consideration showed *S. aureus* isolated that were detected with intermediate susceptibility in a range of 10 to 32% (Table 1). The microorganisms were mainly resistant to penicillin, dicloxacillin, cefotaxime, ampicillin and cephalothin (100%), while they showed resistance to tetracycline and cefuroxime in 81 and 77%, respectively.

The antimicrobial resistance level in this study was seriously high. In the present study, the highest rates of antimicrobial resistance were against to all β-lactam antibiotic group (Fig. 1), which are the most widely used antimicrobials in the treatment of bovine mastitis. Similar results were obtained by Zhang and Buckling (2012) who indicated that 94.8% of the *S. aureus* isolates were resistant to at least one antimicrobial agent, particularly ampicillin and penicillin. León-Galván et al. (2015) and Barboza-Corona et al. (2009) found that 60 to 90% of microorganisms isolated from clinical and subclinical bovine mastitis, were resistant to β-lactam antibiotics. According to Jensen and Lyon (2009) *S. aureus* resistance to multiple antimicrobial agents is driven by the acquisition of mobile genetic elements such as plasmids, transposable genetic elements (insertion sequences and transposons). These genetic elements incorporate preformed antimicrobial resistance determinants and are exchanged between bacterial strains/species via horizontal gene transfer. Since 1940's it was demonstrated the original penicillin-resistant strains through action of β-lactamase, which hydrolyzed β-lactam ring of this antibiotic class. Today, is known that the β-lactamase structural gene (*blaZ*) is present on Tn552-like transposons or remnants thereof. These elements are commonly carried by β-lactamase/heavy-metal resistance plasmids. Chromosomal-mediated resistance could result from integration of plasmids carrying Tn552-like elements or from only insertion of these elements (Jensen and Lyon 2009).

In Mexico, it is very common that, at the end of period of lactation in dairy cattle, farmers use a prophylactic dose of antibiotics (i.e., penicillin and cephalosporins) into the udder (León-Galván et al. 2015). Although the use of antibiotics to prevent mastitis is a common practice, it is obvious that this procedure might generate penicillin-resistant microorganisms (Rushton et al. 2014). Furthermore, uncontrolled use of antibiotics for therapeutic and nutritive purposes has brought about the emergence of pathogenic bacteria resistant to most of currently available antimicrobial agents (Brnakova and Godany 2005). Antibiotic resistance in *S. aureus* is also a growing concern, with the overall rates of antimicrobial resistance in bovine *S. aureus* isolates varying widely by region (Makovec and Ruegg 2003; Pitkala et al. 2004; Sabour et al. 2004). The main explanation of this is because the treatment of *Staphylococcus* infections, which is largely based on antibiotics, bacteria are increasingly acquiring resistance to commonly used drugs (Zhang et al. 2016). More than a decade ago, the Center of Disease Control (CDC) (2008) reported that approximately 95% of hospital staphylococcal ('staph') infections failed to respond to first-line antibiotics. The need for a better knowledge about encoded bacterial resistance systems, their prevalence and genetic variation, and establishment of collections of bacteria containing as large part of this variation it is very important (Khan and Nilsson 2015). Therefore, this suggests that genetic background and their susceptibility pattern may be considered for the design of modern strategies to control of *S. aureus* infections in the dairy farms (Barrera-Rivas et al. 2017).

Bacteriophage susceptibility

The effect of phage infection on isolates obtained in this study was observed in the 100% of *S. aureus* samples by mean display of clear zone of inhibition. It was showed a global mean of 17.5 mm of inhibition diameter (Table 2). The lytic activity of bacteriophages solution seems like more suitable used in this study showed a variable range of inhibition diameter from 10.4 to 23.3 mm according to different factor and levels evaluated, however all of them registered significant differences ($p < 0.01$) (Table 2). It is necessary to emphasize that the solution that registered the widest inhibition halo (23.3 mm) was phage prepared solution corresponding to the highest grade of purification (lysate solution No. 3, propylene glycol excipient 3, high concentration (0.8 mL), and alkaline pH (8.0) ($p < 0.000$). The development of clear zones of lysis against *S. aureus* using specific phage lysate, clearly indicated that the phage was lytic. These in vitro results indicated that the use of bacteriophage to control *S. aureus* could be an alternative for bovine subclinical mastitis cases, which should be tested in further studies.

One of the alternative approaches of how eliminate pathogens from the mammary gland is the use of bacteriophages (Brnakova and Godany 2005). Several studies carried out in in vitro, murine and in vivo or clinical assays have been

Table 1 *S. aureus* strains isolated from dairy cattle and their susceptibility to antibiotics

S. aureus	Antibiotics											
Number	PE	DC	PEF	CXM	GE	CTX	SXT	TE	AM	E	CAZ	CF
SA1	R	R	S	I	S	R	S	R	R	S	R	R
SA2	R	R	S	R	I	R	R	R	R	S	R	R
SA3	R	R	I	R	R	R	R	R	R	S	R	R
SA4	R	R	S	R	R	R	R	R	R	I	R	R
SA5	R	R	I	R	R	R	R	R	R	R	R	R
SA6	R	R	R	R	R	R	R	R	R	R	R	R
SA7	R	R	I	R	S	R	R	R	R	R	R	R
SA8	R	R	S	R	S	R	S	R	R	S	R	R
SA9	R	R	S	R	S	R	S	R	R	I	R	R
SA10	R	R	I	R	R	R	R	R	R	S	R	R
SA11	R	R	S	R	S	R	I	R	R	R	R	R
SA12	R	R	S	R	S	R	I	R	R	S	R	R
SA13	R	R	I	R	I	R	R	R	R	S	R	R
SA14	R	R	S	R	S	R	I	R	R	R	R	R
SA15	R	R	S	R	S	R	S	I	R	S	R	R
SA16	R	R	S	R	S	R	S	I	R	S	R	R
SA17	R	R	S	I	S	R	S	R	R	R	R	R
SA18	R	R	I	R	S	R	S	I	R	I	R	R
SA19	R	R	R	R	R	R	R	R	R	R	R	R
SA20	R	R	R	R	S	R	R	R	R	R	R	R
SA21	R	R	S	S	S	R	S	S	R	S	R	R
SA22	R	R	R	R	R	R	R	R	R	R	R	R
SA23	R	R	S	R	I	R	I	R	R	R	R	R
SA24	R	R	S	R	R	R	R	R	R	R	R	R
SA25	R	R	I	R	S	R	S	R	R	R	R	R
SA26	R	R	S	R	S	R	R	R	R	S	R	R
SA27	R	R	S	R	S	R	S	I	R	S	R	R
SA28	R	R	S	I	S	R	I	R	R	R	R	R
SA29	R	R	R	R	R	R	R	R	R	R	R	R
SA30	R	R	I	R	S	R	R	R	R	R	R	R
SA31	R	R	S	S	S	R	S	S	R	S	R	R
SA32	R	R	S	I	S	R	S	R	R	S	R	R
SA33	R	R	S	R	I	R	R	R	R	S	R	R
SA34	R	R	I	R	R	R	R	R	R	S	R	R
SA35	R	R	S	R	R	R	R	R	R	I	R	R
SA36	R	R	I	R	R	R	R	R	R	R	R	R

R resistant, *S* susceptible, *I* intermediate, *PE* Penicillin, *DC* dicloxacillin, *PEF* pefloxacin, *CXM* cefuroxime, *GE* gentamicin, *CTX* cefotaxime, *SXT* sulfamethoxazole-trimethoprim, *TE* tetracycline, *AM* ampicillin, *E* erythromycin, *CAZ* ceftazidime, *CF* cephalothin

demonstrated important results by means the high efficacy of the bacteriophages on *S. aureus* isolated from bovine mastitis (Capparelli et al. 2007; Kwiatek et al. 2012; Schmelcher et al. 2012; Mishra et al. 2013; Hamza et al. 2016; Becker et al. 2016; Fan et al. 2016). Similar results were obtained for staphylococcal isolates from bovine mastitis and human's hand samples with high frequency of antibiotic resistance obtained from small farms in the highlands of Central Mexico, which were tested against members of two phage families [*Siphoviridae* (phi11, phiIPLA88 and phiIPLA35) and *Myoviridae* (phiIPLA-RODI and phiIPLA-C1C)]. The in vitro effectivity of bacteriophage to infect and lyse the *S. aureus* isolates, showed and important percentage of efficacy of inhibition growth of *S. aureus* isolated; however, it was mentioned a phage-resistance event from two *S. aureus* human's hand isolates (Salgado-Ruiz et al. 2015).

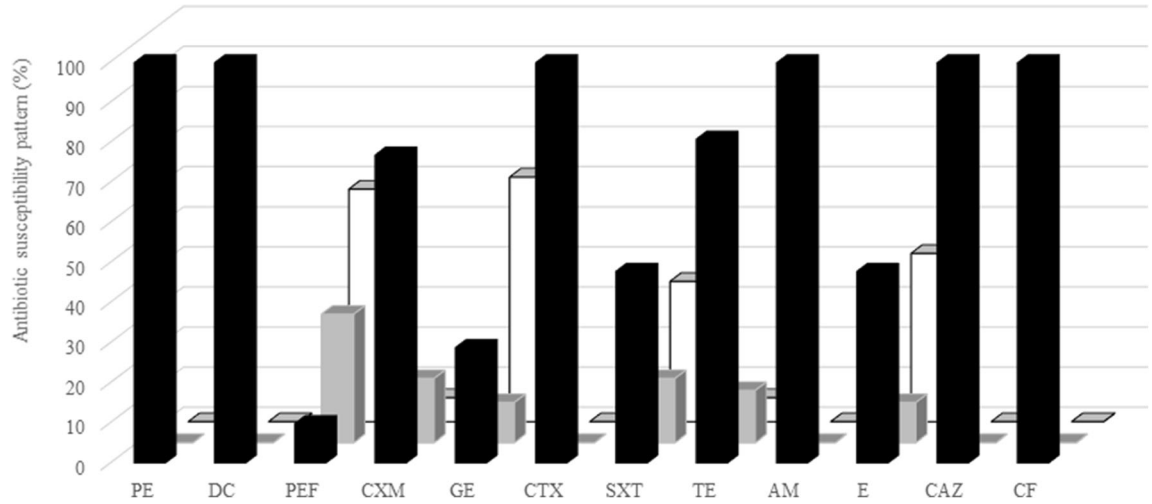

Fig. 1 Percentage of sensitivity in vitro by standard disk diffusion (BioRad®) of different antibiotics against to *S. aureus* strains isolated from bovine mastitis. Graphic bars represent the percentage of sensitive (white), intermediate (grey), or resistant (black). Penicillin (PE), dicloxacillin (DC), pefloxacin (PEF), cefuroxime (CXM), gentamicin (GE), cefotaxime (CTX), sulfamethoxazole-trimethoprim (SXT), tetracycline (TE), ampicillin (AM), erythromycin (E), ceftazidime (CAZ), cephalothin (CF)

Zoonotic transfer of pathogenic bacteria, either through direct contact or via the food chain, represents a serious threat to public health (Boss et al. 2015), which is also particularly true for zoonotic pathogens such as *S. aureus* that may infect humans (Chroboczek et al. 2013) or that display resistance to antimicrobials used in humans (Garcia-Alvarez et al. 2011; Leverstein-van Hall et al. 2011). The treatment of bacterial infections could benefit from our extensive knowledge of the genetics and evolution of antibiotic resistance, coupled with a promising alternative therapeutic agent that could act as a powerful enhancer (Torres-Barceló and Hochberg 2016).

Conclusion

The pathological role of *S. aureus* in bovine mastitis has increased inevitably and there is an urgent need to highlight the threat to both animal and human health. However, the in vitro susceptibility of *S. aureus* isolated from subclinical mastitis cases to phage infection was of 100% of efficacy in this study. Alternative approaches based on bacteriophages has proved to be useful as potential tool to eliminate the pathogen from cattle, particularly, the several problems associated with the control of bovine mastitis using phage therapy and can

Table 2 Antimicrobial susceptibility of 27 *S. aureus* strains isolated from subclinical bovine mastitis by Spot test using three bacteriophage prepared solution

Factor Global mean	Level All	Number 243	Mean 17.5761	Lower limit	Upper limit	*P*-value
Lysate	1	81	13.0	11.3644	14.6356	0.000
	2	81	18.4198	16.7842	20.0553	
	3	81	21.3086	19.6731	22.9442	
Excipient	1	81	18.3333	16.6978	19.9689	0.000
	2	81	21.8272	20.1916	23.4627	
	3	81	12.5679	10.9323	14.2035	
Concentration	1	81	10.4568	8.82123	12.0923	0.000
	2	81	18.8889	17.2533	20.5244	
	3	81	23.3827	21.7472	25.0183	
pH	1	81	14.963	13.3274	16.5985	0.006
	2	81	18.5432	16.9077	20.1788	
	3	81	19.2222	17.5867	20.8578	

Lysate (three purified phages level), excipient (1: saline solution, 2:PBS and 3:propylene glycol), phage concentration (1: 0.3, 2:0.5 and 3:0.8 mL) and pH (1:5, 2:7 and 3:8)

Means by least squares (with 95.0% confidence intervals)

represent an alternative to conventional antibiotic control of multi-resistance bacterial infections. Our in vitro studies showed encouraging results but more tests, namely in vivo, are still critical and this is probably a result of the shortage of more in-depth research.

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Compliance with ethical standards

Animal studies Animal studies were carried out humanely and according to national and international Animal Care and Use Committee protocols.

Conflict of interests All the authors declare no conflict of interest regarding this manuscript.

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