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# Isolation of multidrug resistant coliforms and their bacteriophages from swine slurry

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**Abstract:** Slurry management is a contemporary agricultural problem. Although liquid manure is a valuable fertiliser, it may pose a microbiological threat due to spreading of bacteria resistant to various antibiotics. Fermentation has been proposed as a solution to this problem, although it does not fully eliminate the antibiotic-resistant strains. The presence of gram-negative rods resistant to antimicrobials may cause an uncontrolled transmission of resistance factors and genes in the natural environment. Therefore, a biocontrol system is necessary to prevent this phenomenon. The aim of this study was to detect multidrug-resistant (MDR) bacteria and their lytic bacteriophages in raw swine slurry. For that purpose, MDR bacteria from the family *Enterobacteriaceae* were isolated and tested with bacteriophages using a modified detection method. The study suggests that the bacteriophages present in the slurry are active against MDR bacteria and possibly could be used as a biocontrol agent for reduction of these microorganisms in fertilisers of animal origin. In the scope of the results obtained, a novel approach for the evaluation of slurry based on the preselection method is proposed. Moreover, the study reports the first case of isolation of a bacteriophage active against *Providencia alcalifaciens*.

**Key words:** Antibiotic resistance, bacteriophages, opportunistic pathogens, slurry evaluation

## 1. Introduction

Slurry is considered the best fertiliser due to its content of minerals such as magnesium, phosphorous, and potassium. Moreover, liquid manure includes nitrogen, which, in its organic and mineral forms, is one of the most important plant nutrients (1).

Although the use of slurry is beneficial in agriculture, its application may be considered a threat to the natural environment due to the fact that this material contains microorganisms regarded as opportunistic pathogens. For example, heavy rainfall following the application of slurry may lead to the contamination of water bodies (2). It has been also reported that the survival of multidrug resistant bacteria is widespread in agriculture (3). Formerly, the use of antibiotics in animal production was considered beneficial in terms of production performance (antibiotic growth-promoters). Nevertheless, it also promoted the survival of microorganisms resistant to one or several antibiotics (4). Since 2004, the use of antibiotics in animal husbandry has been prohibited and limited in the European Union only to clinical purposes (5).

Antibiotic resistance is likely to have its origin in environmental, nonpathogenic bacteria. These microorganisms can transmit resistance genes through horizontal gene transfer (HGT) to pathogenic or opportunistic strains. There are growing concerns that nonclinical ecosystems may contribute to the problem of growing resistance (6). Semenov et al. (7) showed evidence that enterohaemorrhagic *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium can be found and survive in soil contaminated with manure or slurry. Moreover, bacteria present in organic fertilisers may bring genes responsible for drug resistance. Reports show that strains of *Salmonella* spp. and *E. coli* may not only survive in this environment but also contain genes making them resistant to several medically important antibiotics such as norfloxacin, ampicillin, or extended-spectrum cephalosporins (7,8). Thus, it has been proven that slurry is a reservoir of antibiotic resistance genes (7).

Bacteria are known for exchanging genetic information via the HGT and absorbing free DNA from the environment. Therefore, it seems highly possible that the resistance genes may spread in the environment, together with pathogenic and opportunistic bacteria. Gao et al. (9) indicated that the use of swine manure on agricultural fields may contribute to spreading of β-lactamase-producing *E. coli*. The prevalence of such microorganisms may be considered a threat to farm animals, staff, and the natural environment. Furthermore, genes of the antibiotic resistance or bacteria containing such genetic material

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may enter other environments as well as food chains, e.g., by contaminating vegetables (3). Van Elsas et al. (10) noted that *E. coli* is able to spread and survive in different open environments such as soil, slurry, and water (10,11).

There is a necessity for an alternative method that would prevent MDR bacteria from spreading in the natural environment. On the other hand, it is known that apart from bacteria, slurry contains bacteriophages that act against them (12). Theoretically, if the quantity of phages in the environment increases it results in a decrease in the number of bacteria. The greatest advantage of using bacteriophages is their specificity against bacteria. This leads to the idea of creating a bacteriophage-based solution to remove pathogenic and opportunistic bacteria from slurry, without killing environmental bacteria that have positive impact on the environment. The idea of such a cocktail was shown by Viazis et al. (12). They successfully isolated bacteriophages active against *E. coli* O157, O26, and O111 from dairy and cattle feedlot manure. Amin et al. (2) simulated the redistribution of, e.g., the phage directed against *S. enterica* serovar Typhimurium in soil treated with slurry. Their results indicate that phages may spread in the environment depending on the soil type. In our other study we were able to eradicate *S. enterica* serovar Enteritidis directly in slurry samples by the addition of bacteriophage sall_v01 (13). On the other hand, bacteria do not remain defenceless in the face of the threat caused by bacteriophages. It is known that microorganisms developed defence mechanisms protecting them before phages, although they differ from resistance to antibiotics (14). For that reason, an application of a cocktail containing bacteriophages will not contribute to the spread of antibiotic resistance in bacteria.

In the scope of the preceding arguments, the slurry evaluation should include the identification of multidrugresistant bacteria (MDR), as well as methods for their removal, based on bacteriophages. Therefore, the goal of the present study was to detect the MDR bacteria belonging to the family *Enterobacteriaceae* in raw swine slurry and to find (in the same environment) bacteriophages specific against these bacteria. An additional goal was to verify whether the applied preselection method is useful for the detection of MDR coliforms in slurry.

## 2. Materials and methods

### 2.1. Materials

Twenty samples of raw pig slurry (from each of two farms) were used as the primary material for the experiments. The liquid manure was collected from two pig farms located in West Pomeranian Voivodeship in Poland, courtesy of Arkadiusz Pietruszka and Michał Grudziński from the Department of Pig Breeding, Animal Nutrition and Food, West Pomeranian University of Technology, Szczecin, Poland. According to the farm owners, antibiotics were occasionally used for the veterinary purposes during animal production. Further information about the farms cannot be given due to provisions from the nondisclosure agreement. Liquid manure was used to isolate bacteriophages and bacterial strains showing features characteristic for the family *Enterobacteriaceae*.

### 2.2. Preselection of bacteria

Bacteria were primarily treated in mixed cultures under selective pressure of antibiotics. In this stage, swine slurry was diluted with phosphate-buffered saline (PBS) down to 0.5 on the McFarland scale (measured with densitometer DEN-1; Biosan, Latvia). Afterwards, 100 µL of the suspension were spread on the Mueller–Hinton agar (MH) with sterile swabs. Preselection was performed by the application of antimicrobial disks containing seven antibiotics in a way described in the next section. Plates were incubated at 37 °C for 24 h. Once grown, colonies located closest to the disks were selected and streaked into clean plates, which were incubated at 37 °C for 24 h and subjected to further tests. Subsequently, cell morphology was analysed under an optical microscope (Delta L-1000) in order to select only gram-negative strains for the following procedures.

Basic biochemical characteristics of isolates were examined on selective media designed for the growth of coliforms such as MacConkey agar, Xylose Lactose Desoxycholate agar (XLD), and Simmons citrate agar. The negative control consisted of Chapman agar (for staphylococci; CH; Oxoid) and Kanamycin Aesculin Azide agar (for enterococci; KAA; Sharlau). The isolates were also examined using oxidase (Oxoid) and catalase (hydrogen peroxide, 3% v/v) tests.

### 2.3. Antibiotic susceptibility testing

The antibiotic susceptibility testing was performed according to the Clinical and Laboratory Standards (15) and EUCAST (16) instructions. The following antibiotics (disks; Oxoid) were used for the tests: vancomycin (30 µg; VA), streptomycin (300 µg; S), ampicillin (25 µg; AMP), sulfamethoxazole (100 µg; RL), polymyxin B (300 U; PB), ciprofloxacin (5 µg; CIP), and chloramphenicol (30 µg; C). This particular selection covers antibiotics that act against all of the main functions in bacterial cells, i.e. cell wall synthesis (VA, AMP), translation (S, C), folic acid synthesis (RL), cell membrane integrity (PB), and cell division (gyrase inhibitor) (CIP). The disk diffusion method was applied in order to obtain resistance patterns of the isolates. For this purpose, monocultures were suspended in the PBS to establish optical density equal to 0.5 on the McFarland scale. Prepared suspensions were accurately swabbed into MH agar and the antimicrobial disks were placed. The plates were incubated at 37 °C for 24 h and the halo zones were measured. Zone diameters indicating

susceptibility or resistance were compared with the CLSI standards (15). Due to the lack of guidelines considering sulfamethoxazole, standards for sulfonamides in general were applied. The method was validated according to the producer's guidelines. The results gained during the study were replicable and conducted in triplicate.

**2.4. Biochemical identification**

The isolates showing the highest rate of antibiotic resistance were identified with the use of the RapID One biochemical identification system (Thermo Scientific) for bacteria that belong to the family *Enterobacteriaceae*. The system is an acclaimed tool for identification of microorganisms in medical laboratories. Assays were performed according to the user's manual. The results were compared with the producer's database using the software ERIC (Thermo Scientific).

**2.5. Isolation of bacteriophages**

Slurry samples for bacteriophage isolation were centrifuged (4500 rpm/4 °C/5 min) and supernatant was passed through a 0.22 µm filter. Prepared slurry samples were added to the cultures of previously isolated bacteria during the midexponential growth phase and incubated (12 h/37 °C). The phage lysate was then centrifuged (4500 rpm/4 °C/5 min) and the supernatant was filtered through a 0.2 µm filter. Finally, the plaque assay was performed using a double-overlay method described by Kropinski et al. (17) and a culture of earlier isolated MDR coliforms was used for isolations. Pure isolates of bacteriophages were obtained by single plaque isolation performed in triplicate.

**2.6. Lytic activity of isolated bacteriophages**

The phages were tested in liquid culture of the isolated drug-resistant strains. Tubes of lysogeny broth (LB) were inoculated with each isolate and incubated at 37 °C overnight. The fresh LB medium (supplemented with 10 mmol $CaCl_2$ and $MgSO_4$) was inoculated with 2% overnight culture and incubated to reach $OD_{600nm}$ = 0.2 (midexponential phase). One flask was inoculated with respective phage ($10^9$ PFU (plaque forming unit) × $mL^{-1}$) and the second one was allowed as a control (no phages - phage-free buffer was added). Aliquots were taken every 1 h and the OD was measured at the wavelength of 600 nm using a microplate reader, NanoQuant infinite M200Pro (Tecan).

**3. Results and discussion**

In total, 32 distinctive bacterial strains were isolated during the preselection process. Further monocultures on selective media for the growth of coliforms led to the isolation of 18 strains of morphology and biochemical features matching the family *Enterobacteriaceae*. Experiments confirmed that the bacterial composition of swine slurry is highly diversified (18). Although the slurry suspensions were diluted to 0.5 MF, growth on media containing antibiotics was extensive, which is shown in Figure 1. Therefore, the step of culturing bacteria on medium with antibiotic disks significantly supported the selection of MDR bacteria that were used in further steps.

Among other isolates, eight MDR bacteria belonging to the family *Enterobacteriaceae* were isolated and selected

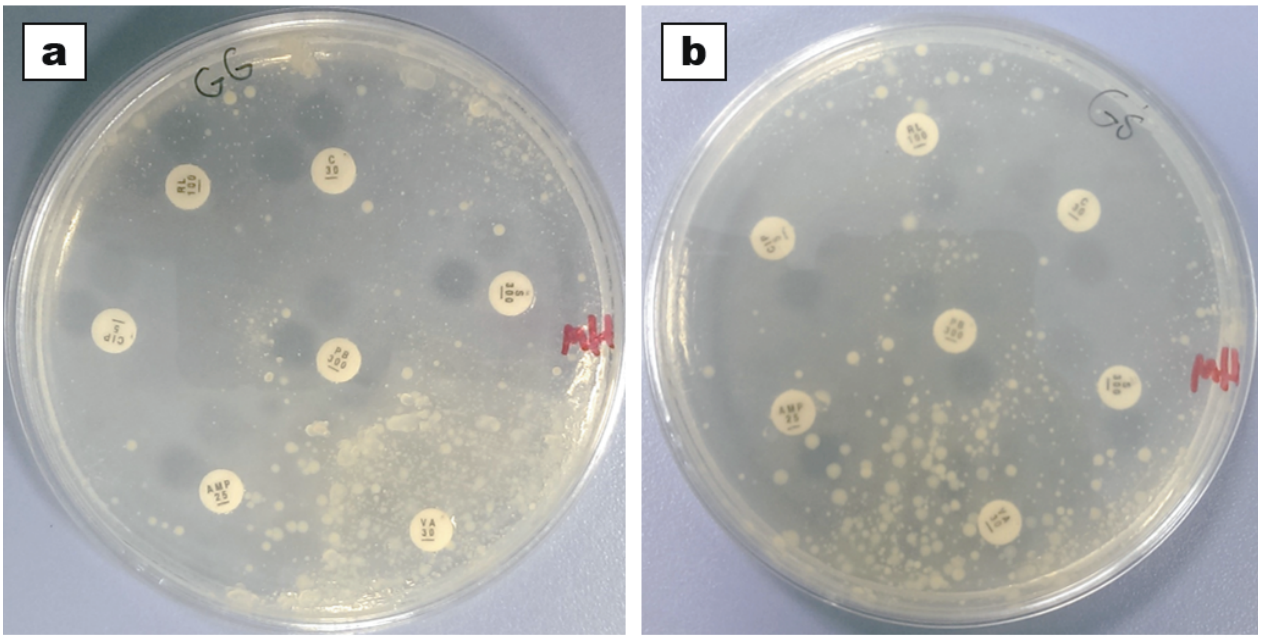


**Figure 1.** High microbial biodiversity on preselection plates (with antibiotic disks) of representative samples from the first (a) and second (b) swine farm.

for further experiments based on their morphology and growth on selective media. The RapID assay was used to identify the following genera: *Escherichia*, *Proteus*, *Providencia*, and *Shigella*. These microorganisms showed the highest rate of resistance and are listed in the Table. This research aimed at selecting MDR bacteria; therefore data obtained with other isolates were omitted. It should be noted that the relative antibiotic resistance (i.e. the presence of MDR in one farm and their absence in the other) detected among strains was not significantly different between the swine farms, which suggests that antibiotic resistance may occur regardless of addition of antibiotics.

*E. coli* strains were the most frequent isolates. The results indicate that raw slurry is a source of multidrug-resistant bacteria, which is in compliance with other publications (7,9,10). These microorganisms collectively carry the resistance to all selected antibiotics that are active against all crucial cell functions. All of the isolated MDR bacteria showed resistance to ampicillin, which may confirm observations made by Fan et al. (19), who reported that this feature was widespread in samples taken from swine farms. Similarly, Rzewuska et al. (20) reported that MDR bacteria, including pathogenic *E. coli*, have been isolated from many companion animals that share the same environment.

In the present study 20 samples of swine slurry were screened for the presence of lytic bacteriophages against isolated MDR bacterial strains. The selection of lytic bacteriophages was completed based on triple passage of phage, positive in vitro multiplication, and cross-checking on the reference strain. Plaques formed by isolated bacteriophages are shown in Figure 2. Phages that could infect and produce plaques on strains 1, 3, 4, and 6 formed small (approx. 1 mm in diameter), clear plaques with absence of halo zones. Phages 2, 7, and 8 produced large (approx. 3.5–4 mm in diameter), clear plaques with distinguishable halo zones, in contrast to the bacteriophage – 5, which formed small (approx. 2.5 mm in diameter) turbid plaques. All bacteriophages were stored in the phage collection of the Department of Immunology, Microbiology and Physiological Chemistry. The fact that phages were isolated from the same environment as bacteria may indicate that these organisms, due to the coevolutionary dynamics between bacteria and the bacteriophage, are in balance, which enables them to co-exist in one environment (21). Based on the available literature, this could be the outcome of dimensional interactions and natural forming of bacteriophage insensitive mutants in the case of the use of only one phage for therapy purposes (22). Nevertheless, isolated bacteriophages successfully infected the studied bacteria.

Isolated bacteriophages could reduce bacterial culture in vitro. The reduction of bacterial growth was correlated with a decrease in optical density of culture that was observed in comparison to the control sample containing phage-free buffer. The highest lytic activities observed for the analysed bacteriophages against isolated MDR bacteria are shown in Figure 3. Interestingly, the increase in OD for bacterial culture was observed for phages 1 and 6. This phenomenon may indicate that the presence of bacteriophages triggered an innate bacterial resistance (associated with derivation of resistant bacterial clones); nevertheless the reduction of bacterial quantity was significant in comparison to the control. Based on these results we assume that it would be advisable to use two or more bacteriophages against every isolate, similarly to results shown by O'Flynn et al. (23) for the bacteriophage

Table. Isolates showing the highest rate of resistance found in the swine slurry.

| No. | Strain | Antibiotic | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | AMP | C | CIP | PB | S | RL | VA |
| 1. | *Escherichia coli* | R | S | I | S | I | R | R |
| 2. | *E. coli* | R | S | S | S | I | R | R |
| 3. | *E. coli* | R | S | R | S | S | R | R |
| 4. | *E. coli* | R | S | R | S | S | R | R |
| 5. | *Proteus vulgaris* | R | S | I | R | S | R | R |
| 6. | *Providencia alcalifaciens* | R | S | I | R | S | I | R |
| 7. | *Shigella* sp. | R | S | I | S | S | R | R |
| 8. | *Shigella* sp. | R | R | S | S | S | R | R |

Legend: 1–8 – strain number; R – resistant, I – intermediate, S – susceptible; AMP – ampicillin, C – chloramphenicol, CIP – ciprofloxacin, PB – polymyxin B, S – streptomycin, RL – sulfamethoxazole, VA – vancomycin.

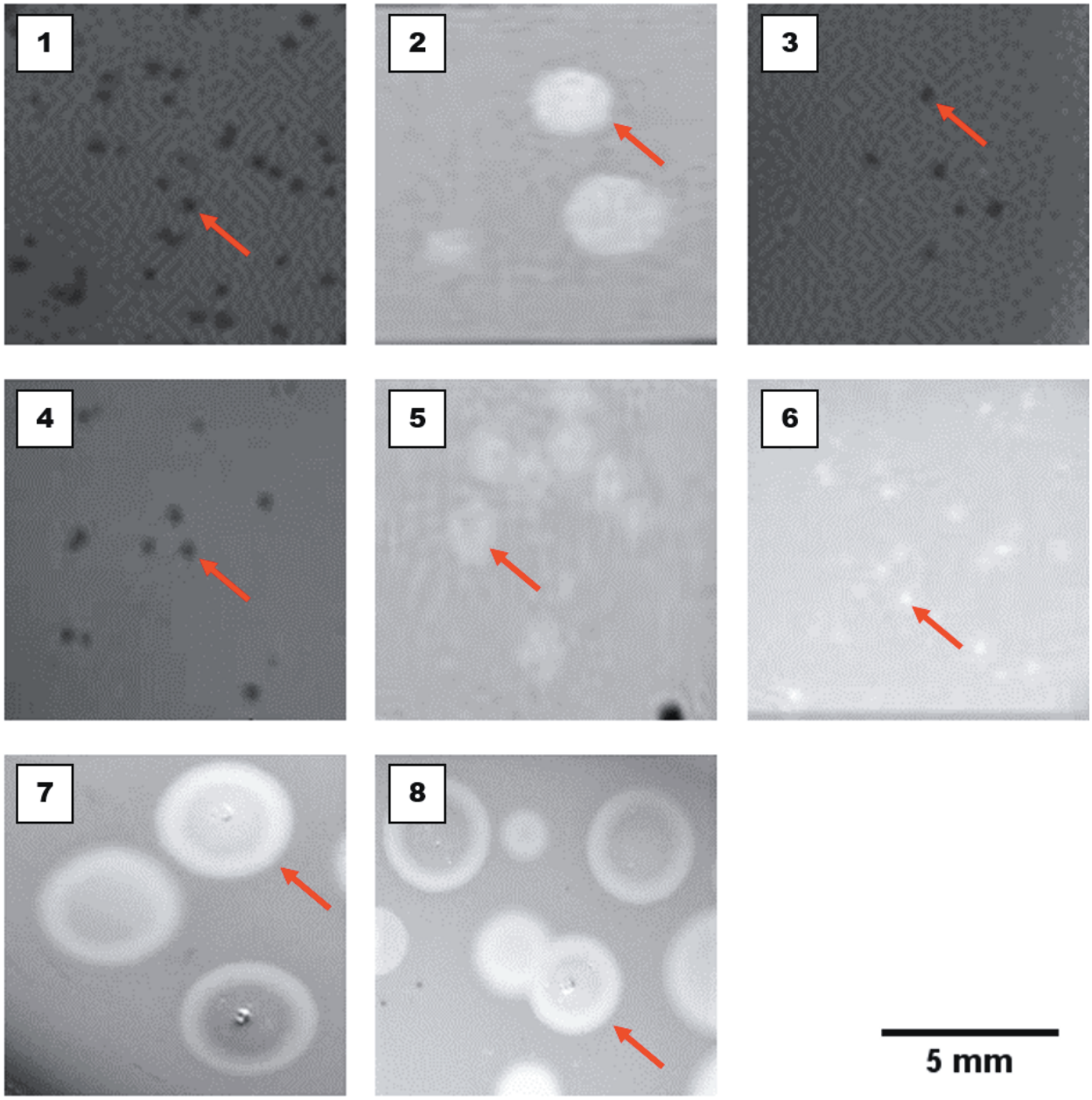


**Figure 2.** Morphology and size of plaques formed by isolated bacteriophages on culture plates; 1–8 – bacterial strains; arrows indicate a representative plaque for each isolate.

cocktail prepared against *E. coli* O157:H7. This is associated with the strengths and shortcomings of phage therapy such as high specificity and possible bacteriophage resistance, in the case of which combined phage use could be necessary. Nevertheless, this study shows that swine slurry, as other faecal samples, has a great potential for the isolation of bacteriophages, which confirms findings reported in other publications (12,24,25), where lytic bacteriophages were also isolated from various slurry samples.

Isolated bacterial strains were collectively resistant to all antibiotics used in this study, but vulnerable to infection by isolated lytic bacteriophages, which indicates that these bacteriophages can be used as a natural biocontrol agent. Furthermore, the use of bacteriophages in slurry management could be (as a consequence) useful in reducing multidrug-resistant bacteria belonging to the family *Enterobacteriaceae*. Nevertheless, the mixture should consist of more than one bacteriophage for each genus that is to be reduced.

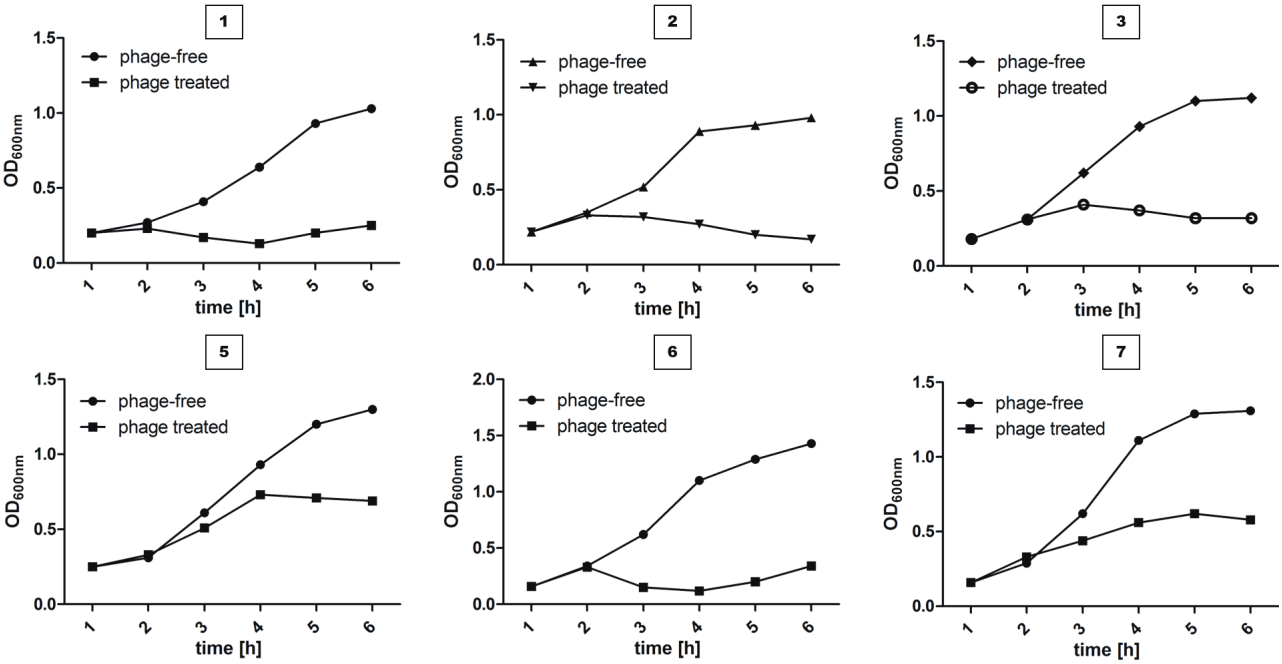


**Figure 3.** Lytic activity of the most active bacteriophages on bacterial strains: 1, 2, 3, 5, 7 – isolate number (the representative experiment results are shown).

Based on the search in the Scopus, NCBI, and Google Scholar databases (on 26.10.2017), this is the first report of the isolation of bacteriophages against *P. alcalifaciens* (strain no. 6). This was a significant discovery due to clinical threats caused by this bacterium. *P. alcalifaciens* can contribute to the development of gastroenteritis in humans and animals (26,27). Bacteriophages active against *Providencia* spp. could be used as biocontrol agents to reduce the transmission of these hazardous microorganisms.

Slurry is an environment in which MDR bacteria can be found along with lytic bacteriophages that are active against them. Simultaneous occurrence of bacteria and their bacteriophages in liquid manure may indicate that these organisms are in balance, enabling either of them to co-exist. Nevertheless, isolated phages are active against MDR bacteria, which is a basis to apply them in further field studies. Additionally, the used preselection technique allowed successful and rapid detection of multidrug resistant coliforms; thus it can be used to inexpensively detect MDR microorganisms in a single laboratory procedure.

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## References

1. Mazur Z, Mazur T. Effects of long-term organic and mineral fertilizer applications on soil nitrogen content. Polish J Environ Stud 2015; 24: 2073-2078.
2. Amin MGM, Si Unek J, Laegdsmand M. Agricultural Water Management Simulation of the redistribution and fate of contaminants from soil-injected animal slurry. Agric Water Manag 2014; 131: 17-29.
3. Reuland EA, al Naiemi N, Raadsen SA, Savelkoul PH, Kluytmans JA, Vandenbroucke-Grauls CM. Prevalence of ESBL-producing Enterobacteriaceae in raw vegetables. Eur J Clin Microbiol Infect Dis 2014; 33: 1843-1846.
4. Chowdhury PR, McKinnon J, Wyrsch E, Hammond JM, Charles IG, Djordjevic SP. Genomic interplay in bacterial communities: implications for growth promoting practices in animal husbandry. Front Microbiol 2014; 5: 394.

5. EC Regulation (EC) No 1831/2003 of the European Parliament and of The Council of 22 September 2003 on additives for use in animal nutrition. European Commission, 2003.

6. Martínez JL. Bottlenecks in the transferability of antibiotic resistance from natural ecosystems to human bacterial pathogens. Front Microbiol 2012; 2: 265.

7. Semenov AV, van Overbeek L, van Bruggen AHC. Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. Appl Environ Microbiol 2009; 75: 3206-3215.

8. Andersen JL, He GX, Kakarla P, Ranjana KC, Kumar S, Lakra WS, Mukherjee MM, Ranaweera I, Shrestha U, Tran T, et al. Multidrug efflux pumps from *Enterobacteriaceae*, *Vibrio cholerae* and *Staphylococcus aureus* bacterial food pathogens. Int J Environ Res Public Health 2015; 12: 1487-1547.

9. Gao L, Hu J, Zhang X, Wei L, Li S, Miao Z, Chai T. Application of swine manure on agricultural fields contributes to extended-spectrum β-lactamase-producing *Escherichia coli* spread in Tai'an, China. Front Microbiol 2015; 6: 313.

10. Van Elsas JD, Semenov AV, Costa R, Trevors JT. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. ISME J 2010; 5: 173-183.

11. Berglund B. Method for fluorescent marker swapping and its application in *Steinernema* nematode colonization studies. J Microbiol Methods 2015; 113: 34-37.

12. Viazis S, Akhtar M, Feirtag J, Brabban AD, Diez-Gonzalez F. Isolation and characterization of lytic bacteriophages against enterohaemorrhagic *Escherichia coli*. J Appl Microbiol 2011; 110: 1323-1331.

13. Grygorcewicz B, Grudziński M, Wasak A, Augustyniak A, Pietruszka A, Nawrotek P. Bacteriophage-mediated reduction of *Salmonella* Enteritidis in swine slurry. Appl Soil Ecol 2017; 119: 179-182.

14. Abedon ST. Bacterial "immunity" against bacteriophages. Bacteriophage 2012; 2: 50-54.

15. CLSI. M100-S25 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement An informational supplement for global application developed through the Clinical and Laboratory Standards Institute consensus process. Clinical and Laboratory Standards Institute 2015.

16. Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect 2014; 20: 255-266.

17. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol 2009; 501: 69-76.

18. Cotta MA, Whitehead TR, Zeltwanger RL. Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. Environ Microbiol 2003; 5: 737-745.

19. Fan Y, Mei LS, Ling HH, Ping C. Heavy metal and antibiotic resistance in bacteria isolated from the environment of swine farms. J Chem Soc Pak 2014; 36: 360-364.

20. Rzewuska M, Czopowicz M, Kizerwetter-Świda M, Chrobak D, Błaszczak B, Binek M. Multidrug resistance in *Escherichia coli* strains isolated from infections in dogs and cats in Poland (2007–2013). Sci World J 2015; 2015: 1-8.

21. Koskella B, Meaden S. Understanding bacteriophage specificity in natural microbial communities. Viruses 2013; 5: 806-823.

22. Sharma M. Lytic bacteriophages: potential interventions against enteric bacterial pathogens on produce. Bacteriophage 2013; 3: e25518.

23. O'Flynn G, Ross RP, Fitzgerald GF, Coffey A. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Appl Environ Microbiol 2004; 70: 3417-3424.

24. Grygorcewicz B, Struk M, Wasak A, Nawrotek P. Effective bacteriolysis of Shiga toxin-producing *Escherichia coli* O157: H7 caused by specific bacteriophage isolated from pig slurry. Acta Sci Pol Zootech 2015; 14: 69-76.

25. O'Flaherty S, Ross RP, Flynn J, Meaney WJ, Fitzgerald GF, Coffey A. Isolation and characterization of two anti-staphylococcal bacteriophages specific for pathogenic *Staphylococcus aureus* associated with bovine infections. Lett Appl Microbiol 2005; 41: 482-486.

26. Vieira ABR, Koh IHJ, Guth BEC. *Providencia alcalifaciens* strains translocate from the gastrointestinal tract and are resistant to lytic activity of serum complement. J Med Microbiol 2003; 52: 633-636.

27. Yoh M, Matsuyama J, Ohnishi M, Takagi K, Miyagi H, Mori K, Park KS, Ono T, Honda T. Importance of *Providencia* species as a major cause of travellers' diarrhoea. J Med Microbiol 2005; 54: 1077-1082.