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Determination of anti-phage antibodies in calf sera following application of *Escherichia coli* and *Mannheimia haemolytica*-specific bacteriophages

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Abstract

Introduction: The widespread occurrence of drug-resistant bacteria has increased interest in alternatives to antibiotics for combatting bacterial infections, among which bacteriophages play an important role. The ability of phage proteins to induce an anti-phage immune response can significantly limit the effectiveness of treatment, which was the basis for the study described in this article. The aim of the study was to assess the effects of bacteriophages on the induction of an anti-phage humoral response in calves. **Material and Methods:** The study was conducted using phage components of experimental preparations and sera from calves treated and not treated with phages. Levels of G, M and A immunoglobulins were analysed by ELISA. The assay plates were coated with whole *Escherichia coli* and *Mannheimia haemolytica* phages and selected phage proteins obtained in sodium dodecyl sulphate-polyacrylamide gel electrophoresis and two-dimensional electrophoresis. Neutralisation of phages by immunoglobulins was assessed by determining phage titres using double-layer plates. **Results:** The results confirmed an increased anti-phage response affecting all immunoglobulin classes in the calf sera. The highest significant ($P \leq 0.05$) level of antibodies was observed for IgG in the sera of calves receiving phages. The phage neutralisation test showed a significant differences ($P \leq 0.05$) in the reduction of phage titres in comparison to untreated calves. **Conclusion:** Despite the induction of an anti-phage response, no significant negative effect on the antibacterial activity of phages was observed *in vitro*.

Keywords: bacteriophages, *E. coli*, ELISA, *M. haemolytica*, immunoglobulins.

Introduction

The widespread increase in drug resistance among bacteria has led to the development of alternative methods to antibiotics to combat microorganisms. In the last decade, bacteriophages have become an important alternative to antibiotics to fight and prevent bacterial infections in people and animals (16). Their high antibacterial efficacy and lack of effect on commensal bacteria, confirmed in numerous experimental phage therapies, provide the basis for further development of their use to treat infections, particularly those caused by multidrug-resistant bacteria (24, 30). One problem potentially limiting the use of bacteriophages in therapy is the possibility of induction of a humoral immune response against phage proteins in the host. As reported by Batinovic *et al.* (4), phage particles consist of nucleic acid and phage proteins forming a coat that protects the genetic material. This structure is unquestionably a factor in the induction of phage-neutralising antibody production, and thus can influence interactions and treatment effects (11, 17).

Some reports (6, 8, 11) also confirm the occurrence of natural phage antibodies, which can be produced in humans and animals as a result of the common occurrence of bacteriophages in the environment. For example, bacteriophages have been detected in wastewater, water bodies, soil, food and animal feed,

and the oral cavity (dental plaque and saliva) and gastrointestinal tracts of humans and animals, as well as in commercial sera or human vaccines. The total number of bacteriophages in the environment has been estimated at 10^{32}, which is ten times the number of the characterised bacteria (25). Dąbrowska *et al.* (7) showed the presence of anti-phage antibodies (anti-T4) in more than 80% of healthy human subjects who had never received phage therapy. Other studies have confirmed elevated concentrations of antibodies against bacteriophages specific for *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains in healthy individuals participating in research using these bacteriophages (5, 33).

Administration of bacteriophages can cause an increase in antibodies against phage proteins. Nevertheless, in some studies in humans (18, 32) and in our own previous research in calves (1), despite a humoral response induced by the application of bacteriophage preparations, a therapeutic effect was observed. The outcomes were a significant reduction or even elimination of bacteria and an improved health status. Moreover, it has been suggested that the presence of anti-phage antibodies does not necessarily lead to complete inactivation of phages but allows them to multiply in bacterial cells and cause their lysis (11). The strength of the humoral response against phage proteins and of their consequent inactivation may be influenced by both the type of preparation used (monophage or a cocktail) and the route of administration, *e.g.* topical, parenteral or oral (18).

Another significant adverse effect of phage therapy is the possibility of an anaphylactic response to foreign phage proteins. However, this type of reaction has been shown to be infrequent; bacteriophages may induce an increase in the expression of the anti-inflammatory interleukin (IL)-1 receptor antagonist (IL-1RA) and stimulate IL-10, an anti-inflammatory cytokine that blocks the expression of pro-inflammatory cytokines such as IL-1 and IL-6 and inhibits the activity of Th1 cells, NK cells and macrophages (10).

The predominant health problems in calves in feedlot and dairy production are respiratory and gastrointestinal diseases, both of which affect animal welfare and economic outcomes in cattle production, causing losses because of mortality and treatment and future production costs. Estimates of the prevalence of bovine respiratory disease induced by *Mannheimia haemolytica* strains in cattle less than one year old have ranged from 4% to more than 80% (23). Diarrhoea is a major health problem in cattle, especially in young calves, causing high morbidity and mortality rates and diminished growth rates and requiring prolonged treatment. Bacteriological examination has confirmed the presence of pathogenic *E. coli* strains of various serovars, including the enterotoxigenic *E. coli* K99, in 46.4% of calves in developing countries (21). In developed countries, the prevalence of K99 or the enteropathogenic *E. coli* (EPEC) O159:H7 strain has been estimated at 2.65–15% (2). The difficulties in treating infections induced by *M. haemolytica* and *E. coli* are due to the long duration of treatment and the lack of an appropriate antibiotic. The widespread use of antibiotic treatment can lead to immunosuppression in calves, increasing their susceptibility to infection, and can also increase bacterial resistance, making it more difficult to effectively eliminate infections. The prevalence of antibiotic-resistant bacteria causing neonatal diarrhoea and respiratory disease in calves has become a serious problem in the control of infection.

The aims of the study were to assess the extent of any induction of a specific anti-phage humoral immune response by selected bacteriophage components of experimental phage preparations used in calves and to assess the preservation of their antibacterial properties. This was prompted by the significant health problems in cattle caused by *M. haemolytica* and *E. coli* infections, as well as by the possibility of induction of an immune response directed against bacteriophage proteins and inhibition of the effects of phage therapies.

Material and Methods

Bacteriophages. Bacteriophages for coating ELISA plates were obtained from the proprietary collection of the Department of Veterinary Prevention and Avian Diseases of the University of Life Sciences in Lublin, Poland. Three of these were φ 26, 27 and 29 phages specific for pathogenic strains of *E. coli* isolated from diarrhoea in calves (1), which were used in an experimental probiotic + phage preparation to combat diarrhoea in these animals (31). The other three were φ 25, A2 and A5 phages specific for strains of *M. haemolytica* serotype 1 isolated from calves with symptoms of the BRD complex (27, 29), which were components of an experimental phage preparation for controlling respiratory infections in calves (26). In the studies cited above, calves at various ages from birth to 5 months received experimental phage preparations. In the case of preparations containing bacteriophages specific for pathogenic *E. coli* strains, the phage titre was 10^9 plaque-forming units (PFU)/mL and in the preparation containing a cocktail of phages specific for *M. haemolytica* strains, the titre was 10^6 PFU/mL. Phages were purified using endotoxin affinity columns. The estimated level of lipopolysaccharides (LPS) in the suppositories by which the preparations were administered, was between 10 and 25 endotoxin units (EU)/mL as determined in the Limulus amebocyte lysate test. A *M. haemolytica* phage cocktail suspended in sea water had a level below 12 EU/mL.

Serum samples. Serum samples for analysis were obtained from calves (n = 8 for each group) that had received experimental preparations of phages for five days. The phages originated from the collection owned by the Department of Veterinary Prevention and Avian Diseases of the University of Life Sciences in Lublin, Poland. The first preparation contained a cocktail with φ 26, 27 and 29 bacteriophages specific for *E. coli* and

was applied *per rectum* in the form of suppositories, and the second contained a cocktail with φ 25, A2 and A5 phages specific for *M. haemolytica* strains and was applied directly to the nasal cavity. Blood from the calves was collected into ethylenediaminetetraacetic acid (EDTA)-free tubes for sera and tubes with EDTA for plasma on days 1, 3, 7 and 11 after the first application of the bacteriophage preparations. Sera and plasma samples were kept at −20°C until analysis.

The control sera were obtained from calves that had not previously been treated with bacteriophages (1, 31), as well as from healthy calves which had not previously had contact with the bacteriophages used in the experimental treatments. All tested sera were coming from the collection owned by the Department of Veterinary Prevention and Avian Diseases of the University of Life Sciences in Lublin. Foetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) was additionally used as a negative control.

Preparation of phage proteins and sodium dodecyl sulphate–polyacrylamide gel electrophoresis. For protein extraction, a 0.5 mL suspension of bacteriophages was treated with 96% ethyl alcohol and incubated for 24 h at −20°C. Following removal of the ethanol layer, the precipitated protein was resuspended in 100 µL of single-strength sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) sample lysis buffer (62.5 mM Tris-HCl, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue, pH 6.8) and heated at 100°C for 10 min. The protein concentration after extraction was determined using Bradford's reagent (Sigma-Aldrich). The gel electrophoresis was performed according to Laemmli (15). Electrophoretic separation was carried out using a molecular weight range from 10 to 200 kDa, after which the gels were stained with Coomassie blue (Sigma-Aldrich). The molecular weight reference was an unstained PageRuler 10–240 kDa protein ladder (Thermo Fisher Scientific, Waltham, MA, USA). The stained electropherograms were analysed with a densitometer in Quantity One software (Bio-Rad, Hercules, CA, USA150 Ge). A two-dimensional protein analysis (2D electrophoresis) was performed according to a previous study (29). Immediately before 2D-PAGE analysis, 50 µg of phage protein from the samples was dissolved in 125 µL of buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio] -1-propanesulfonate and 30 mM Tris (pH 8.8). The weight standard was the 7–240 kDa Perfect Color protein ladder (EURx, Gdańsk, Poland).

Phage ELISA test. The ELISA assay was carried out using 96-well flat-bottom plates (Nunc Maxisorp; Thermo Fisher Scientific). A suspension of phages purified on 0.7% Luria-Bertani agar plates (29) for use as antigens was diluted in 0.05 M coating buffer (carbonate-bicarbonate buffer; Sigma-Aldrich) immediately before use to obtain a titre of 10^8 PFU/mL in the case of whole bacteriophages.

For coating of plates with selected phage proteins, the electrophoresis-derived proteins were eluted using the ReadyPrep Protein Extraction Kit (Total Protein – Bio-Rad) according to the manufacturer's instructions. Then the proteins were separated in 2D electrophoresis and characterised by matrix-assisted laser desorption/ionisation–time of flight mass spectrometry (MALDI-TOF) (29). Selected proteins of the phages specific for *E. coli* and *M. haemolytica* strains, which were of similar molecular mass and pH, were eluted from the 2D gels using the ReadyPrep 2D Cleanup Kit (catalogue nos 163–2130 and 163–2140; Bio-Rad), according to the manufacturer's instructions. The resulting suspension was diluted in the same 0.05 M coating buffer to a concentration of 10 µg/mL directly before use.

Antigen in the form of 100 µL of purified phage preparations was used to coat the 96-well flat-bottom microplates, which were incubated overnight at 4°C. The same procedure was used to coat the plates with phage proteins. Then they were washed five times with 350 µL of phosphate-buffered saline (PBS) with Tween 20 (Tween 20 = 0.1%), and 200 µL of blocking protein (1% solution of casein sodium salt from bovine milk in PBS; Sigma-Aldrich) was applied to the microplate wells to block any non-specific binding sites. The plates were incubated for two hours with 3% skim milk at room temperature, after which phages were added for binding. The microplates were then incubated again for 1 h at 37°C. Bovine serum samples were used as primary antibodies and were diluted in blocking solution with the addition of 0.05% Tween 20 in proportions of 1:1000 and 1:10,000. Each serum sample was applied in duplicate by pipetting 100 µL of the solution to each well. The microplates were loaded with 100 µL/well of secondary antibody solution and incubated again for 1 h at 37°C. Specific anti-phage antibodies bound to antigen were detected by goat horseradish peroxidase (HRP)–conjugated secondary antibodies specific for bovine IgG, IgA or IgM (Sigma-Aldrich). All anti-bovine secondary antibodies were diluted in blocking solution with 0.05% Tween 20 in a proportion of 1:5000. After 3 h incubation at room temperature, 200 µL of o-phenylenediamine (Sigma-Aldrich) suspended in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma-Aldrich) was added to each well, and the plates were incubated at room temperature for a minimum of 15 min in the dark. Then the microplates were shaken and immediately read on a multiwell plate reader (BioRad) at 450 nm. Foetal bovine serum was used as a negative control (Sigma-Aldrich).

Serum phage neutralisation assay. The phage neutralisation assay was carried out using calf serum, with neutralisation measured as the speed of inactivation of phages according to Żaczek *et al.* (32). A 450 µL sample of serum from calves that had received phage preparations containing the phage cocktails described above, diluted 1:100, was added to 50 µL of phage cocktail suspension (1×10^6 PFU/mL for *M. haemolytica* phages and 10^9 PFU/mL for *E. coli* phages). Then the sample was incubated at 37°C for 30 min.

The phage inactivation rate was estimated using the following formula proposed by Żaczek *et al.* (32):

$K = 2.3 \times (D/T) \times \log(P0/Pt)$

where:

K – phage inactivation rate,

D –reciprocal of serum dilution,

T – time of onset of reaction in minutes (in this case after 30 min),

P0 – phage titre at the start of reaction,

Pt – phage titre after reaction.

In accordance with the cited study we assumed that $K < 5$ indicated weak neutralisation of phages, $K \geq 5$–$K \leq 18$ indicated moderate neutralisation, and $K > 18$ indicated a high level of phage neutralisation.

Next, the mixture was diluted 100-fold with enriched broth, and the phage titre was determined by the double-layer plate method according to Huff *et al.* (13).

Statistical analysis. Statistical analysis of the ELISA results between groups was carried out by ANOVA, using the Mann–Whitney U test for independent variables. Additionally, the results were confirmed by Duncan's post-hoc test. Wilcoxon's test (for dependent variables) and the Mann–Whitney U test (for independent variables) were used in the neutralisation test. Statistical significance was set at $P < 0.05$. Results are presented as the mean phage inactivation rate K ± standard deviation (18, 32).

Results

Two main phage proteins were obtained with molecular weights of approximately 92.5 and 69 kDa (Fig. 1), shown by the electrophoretic separations in the SDS-PAGE electropherograms.

Analysis of the electropherograms of phage proteins isolated in 2D electrophoresis revealed two main proteins in a pH range of 4.5–5.9. The height of the two most notable spots, evaluated in MALDI TOF analysis, ranged from 39.008 to 78.966 kDa (Fig. 2).

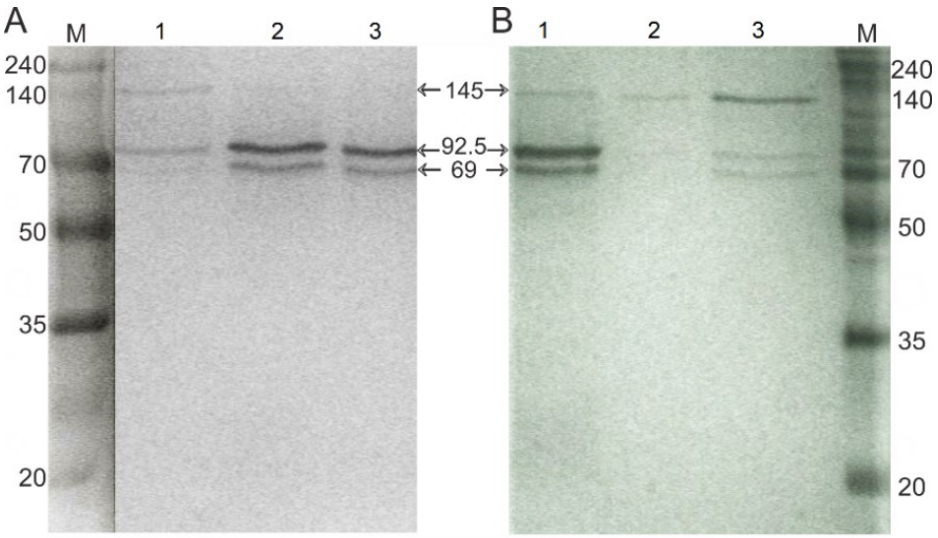

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis electropherograms with phage proteins
A: Lines 1, 2 and 3 – 25 A2 and A5 phages specific for *M. haemolytica* strains; B: Lines 1, 2 and 3 –26, 27 and 29 phages specific for *E. coli*
M –10–240 kDa molecular weight protein ladder

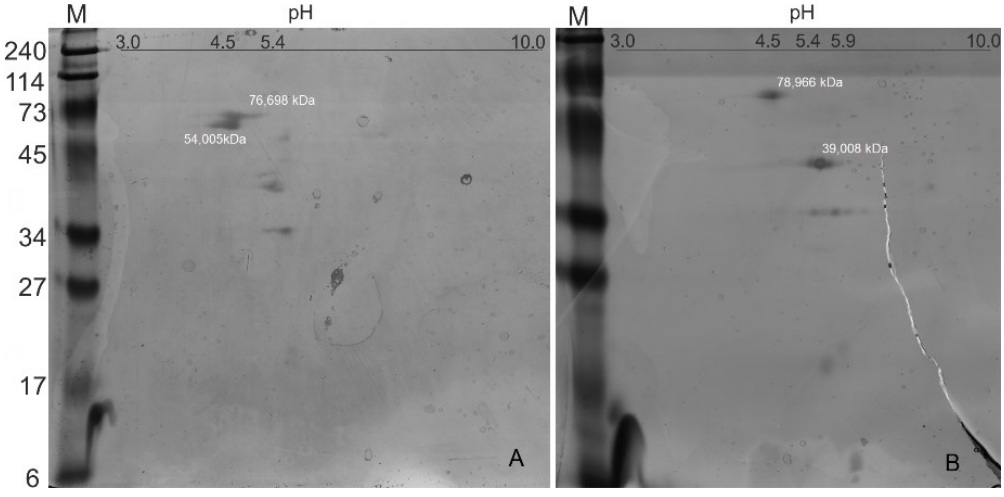

Fig. 2. Two-dimensional electrophoresis of phage proteins
A – phages specific for *M. haemolytica*; B – phages specific for *E. coli*
M – 7–240 kDa molecular weight standard

Table 1. Average absorbance values (±standard deviation) measured in serum samples from calves by ELISA in response to application of bacteriophages specific for *E. coli* and *M. haemolytica*

Parameter	FBS			Calves with clinical signs of disease after phage application			Healthy calves after phage application			Healthy calves not treated with phages		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
Mean level of antibodies against whole *M. haemolytica* and *E. coli* bacteriophages	0.007 ± 0.004	0.008 ± 0.006	0.001 ± 0.0009	0.64 ± 0.09^{ab}	0.192 ± 0.068^{ab}	0.114 ± 0.048^{ab}	0.845 ± 0.111^{ab}	0.239 ± 0.082^{ab}	0.021 ± 0.007^{a}	0.056 ± 0.024^{a}	0.019 ± 0.00^{a}	0.017 ± 0.01^{a}
Mean level of antibodies against whole *E. coli* phages	0.0032 ± 0.001	0.014 ± 0.008	0.001 ± 0.0008	0.74 ± 0.16^{ab}	0.211 ± 0.052^{ab}	0.19 ± 0.09^{ab}	0.71 ± 0.13^{ab}	0.203 ± 0.2^{ab}	0.029 ± 0.003^{a}	0.06 ± 0.009^{a}	0.096 ± 0.038^{a}	0.02 ± 0.007^{a}
Mean level of antibodies against whole *M. haemolytica* phages	0.005 ± 0.004	0.004 ± 0.0001	0.002 ± 0.0005	0.95 ± 0.08^{ab}	0.99 ± 0.111^{ab}	0.23 ± 0.005^{ab}	0.6 ± 0.1^{a}	0.33 ± 0.02^{ab}	0.02 ± 0.002^{a}	0.72 ± 0.017^{a}	0.08 ± 0.03^{a}	0.03 ± 0.001^{a}
Mean level of antibodies against *M. haemolytica* and *E. coli* extracted proteins	0.005 ± 0.003	0.009 ± 0.007	0.001 ± 0.0005	0.71 ± 0.134^{ab}	0.18 ± 0.34^{ab}	0.15 ± 0.05^{ab}	0.53 ± 0.13^{ab}	0.226 ± 0.13^{ab}	0.03 ± 0.016	0.06 ± 0.01^{a}	0.07 ± 0.04^{a}	0.02 ± 0.0009^{a}
Mean level of antibodies against *E. coli* extracted proteins	0.004 ± 0.003	0.002 ± 0.0009	0.001 ± 0.0001	0.83 ± 0.032^{ab}	0.21 ± 0.004^{ab}	0.201 ± 0.002^{ab}	0.68 ± 0.15^{ab}	0.21 ± 0.004^{ab}	0.099 ± 0.007^{a}	0.062 ± 0.001^{a}	0.042 ± 0.002^{a}	0.02 ± 0.008^{a}
Mean level of antibodies against *M. haemolytica* extracted proteins	0.007 ± 0.004	0.004 ± 0.002	0.001 ± 0.000	0.77 ± 0.13^{ab}	0.3 ± 0.04^{ab}	0.15 ± 0.03^{ab}	0.65 ± 0.21^{ab}	0.26 ± 0.18^{ab}	0.05 ± 0.02^{a}	0.071 ± 0.04^{a}	0.05 ± 0.02^{a}	0.03 ± 0.01^{a}

FBS – foetal bovine serum; Ig – immunoglobulin; ^{a} – significant differences ($P \leq 0.05$) in comparison to control (FBS); ^{b} – significant differences ($P \leq 0.05$) in comparison to sera from calves not treated with phages

Table 2. Average values obtained in the phage neutralisation assay

Type of phage solution	Mean K ± SD in untreated calves	Mean K ± SD in treated calves
E. coli phages	0.0019 ± 0.001	7.28 ± 0.54*
M. haemolytica phages	0.0046 ± 0.003	5.57 ± 1.81*
E. coli extracted proteins	0.00156 ± 0.0004	6.74 ± 0.5*
M. haemolytica extracted proteins	0.0013 ± 0.0005	5.053 ± 1.53*

K – phage inactivation rate; SD – standard deviation; * – Wilcoxon's test $P \leq 0.05$

ELISA assay. The results of the ELISA assay showed a lack of anti-phage antibodies in the commercial FBS to either the whole bacteriophages specific for strains of *M. haemolytica* and *E. coli*, or the selected phage proteins. This applied to all tested immunoglobulins (IgG, M and A) (Table 1).

The highest concentrations of anti-phage antibodies were observed for IgG in the sera of calves that had received phages specific for *E. coli* and *M. haemolytica*. The values were statistically significantly different ($P \leq 0.05$) to the values obtained for FBS and for healthy calves that had not received phage treatment. A statistically significantly ($P \leq 0.05$) higher level of antibodies to phages specific for *M. haemolytica* strains was observed in the sera of healthy calves that had not received bacteriophages (Table 1). The values obtained based on the absorbance of other sera were not statistically significantly different. The IgM levels in the sera of sick and healthy calves that had received experimental phage preparations were generally comparable. However, the IgM levels shown by absorbance for phages specific for *M. haemolytica* were disparate and that of sick calves was higher, and these IgM levels in both these groups were statistically significantly ($P \leq 0.05$) higher than those in the other groups of calves and the control (FBS). Absorbance values indicating low levels of IgM antibodies observed in the sera from healthy calves that had not received phage preparations were comparable to the values obtained for FBS (Table 1). The IgA levels were highest and statistically significant ($P \leq 0.05$) in the sera from sick calves that had received experimental phage preparations, as the samples' absorbance indicated. The values were statistically significantly different to those of all groups of calves and the control (FBS). Sera from healthy calves treated with phages and sera from healthy calves not treated with phages yielded similar absorbance values.

Phage neutralisation assay. The assay of bacteriophage neutralisation in sera from calves that had received phage preparations showed significant differences ($P \leq 0.05$) in the reduction of phage titres in comparison to untreated calves. In the comparative analysis between whole phages and phage proteins, despite absolute differences, the values obtained were not statistically significant (Table 2).

Discussion

The results of the study confirm that bacteriophages can induce the production of anti-phage antibodies (mainly IgG and IgM) in calves, which can contribute to inactivation of phages. A statistically significant ($P \leq 0.05$) increase in the antibody level relative to the control (foetal bovine serum) was observed in all tested sera obtained from calves that had received bacteriophages and those that had no contact with the phages contained

in the experimental phage preparations. The results are similar to those obtained by Żaczek *et al.* (32), who applied a cocktail containing staphylococcal MS-1 phages at titres of 5×10^5 PFU/mL and 1×10^9 PFU/mL in patients infected with staphylococci including MRSA and obtained a statistically significant increase in serum titres of IgG and IgM anti-phage antibodies.

The differences in absorbance observed in the present study for different immunoglobulin classes indicate that the phages had varied effects on immune response mechanisms in the calves. The lack of significant differences in IgA levels between the sera may indicate that the immunomodulatory effect of the phages was small or that the immune response was diffused among the large pool of antigens involved in immunomodulation. In a study by Majewska *et al.* (19), oral administration of A3R or 676Z phages specific for *Staphylococcus aureus* to mice in drinking water at a titre of 4×10^9 PFU/mL resulted in very low secretory IgA antibody titres, which were maintained only as long as the bacteriophages were detected in the faeces. In contrast, a statistically significant increase was observed in class G and M serum antibodies, which persisted up to 22 days after phage application for IgM and 50 days for IgG.

The present study also showed no statistically significant differences ($P \leq 0.05$) in absorbance depending on the route of administration, whether intranasal (*M. haemolytica* phages) or rectal (*E. coli* phages). In both cases the absorbance values were similar for class G and M immunoglobulins. Nevertheless, the small differences observed also indicate that the effect of phages on the immune response may depend on the route of administration.

Irrespective of the route of administration of bacteriophages in various animal species, *i.e.* oral, intranasal or intraperitoneal, phages appeared quickly in the bloodstream and in internal organs involved in immune processes, such as the spleen, liver, and thymus. This could be an important indicator of the level of the induced anti-phage immune response (3, 14).

It also seems interesting that the present study showed the presence of IgG and IgM phage antibodies in the sera of healthy calves that had had no physical contact with the bacteriophage preparations, as they were from other farms. These results may indicate the presence of physiological and possibly commensal, bacteriophages as part of the natural microbiome of the gastrointestinal and respiratory tracts, in part a consequence of the natural occurrence of saprophytic *M. haemolytica* in the oral cavity of cattle and *E. coli* in the intestinal microbiome.

The presence of these bacteria and bacteriophages specific for them in the body may be a physiological factor that naturally induces the production of anti-phage antibodies. The occurrence of "natural" phages within the body has been confirmed by researchers such as Nguyen *et al.* (22), who found that every day about 31 billion bacteriophage particles migrate into the human body *via* transcytosis through the cells of the intestinal epithelium, as well as through lung, liver, kidney and brain cells, influencing the host's immune response mechanisms. However, the routes of migration of phages through epithelial cells are still in question. Apart from transcytosis, other likely routes are translocation of bacteriophages through a damaged epithelial barrier or direct uptake from the intestinal lumen by intestinal dendritic cells (4, 20, 22). In addition, commensal intestinal bacteria such as *E. coli* which contain prophage genetic material are able to produce infectious virions, which make such bacteria capable of out-competing various other bacterial species colonising the gut (9).

The present study showed that bacteriophages had a significant effect on the neutralising potential of calf serum in comparison to this potential of the serum of calves that were not treated with phages. However, no statistically significant differences were found between the immunogenic effects of whole phages and phage proteins. This suggests that phage preparations containing purified phage antigens will not stimulate a stronger immune response, as is also the case with vaccines containing purified antigen fragments of microbes, which additionally require an adjuvant to strengthen the immune response.

The relationship between phage administration and the induction of an anti-phage immune response is currently the subject of numerous studies, which have been presented in a review article by Hodyra-Stefaniak *et al.* (12). Some studies have shown that induction of an immune response depends on factors such as duration of treatment, dosage of phages, and route of administration, as well as on the individual properties of specific bacteriophages (20). A significant effect of the route of administration of phage preparations on the induction of serum anti-phage activity has been demonstrated in studies in humans receiving various phage lysates *per os*, topically, *per rectum*, or both orally and topically in combination (18). The anti-phage activity of sera was shown to be strongest ($K > 18$) following topical or topical + *per os* application. The study also confirmed differences in immunogenicity between bacteriophages, there being much higher activity by *P. aeruginosa* F-8, *S. aureus* 676/Z, *S. aureus* 676/F, *S. aureus* fi 200, and *Enterococcus faecalis* Ent 56 phages. High immunomodulatory activity was observed in that research following the use of a phage cocktail. However, the experiment concerned phage therapy in humans, and the results cannot be directly applied to other species, just as it is difficult to directly apply the results obtained in an animal model to humans (32). Therefore, the effects of bacteriophages on the anti-phage activity of sera should be verified in further studies using models of selected animal species.

The results of the present study also indicate that the induction of an anti-phage response by phages did not significantly affect their antibacterial efficacy. Other measurable effects confirming that the lytic activity of

phages was preserved, as shown in our previous research (1, 28), were an improvement in health status and a protective effect lasting for at least three weeks after the final administration of the phages, as well as a significant reduction in the concentration of pathogenic *E. coli* in the faeces and of *M. haemolytica* in the nasal cavity of calves.

The results of the present study as well as those presented by other authors indicate that bacteriophages can induce a humoral immune response in humans and animals. However, there are differences in the strength of the response induced and the potential effectiveness of phage treatments in fighting infections. Despite differences in the levels of induction of anti-phage antibodies, the effects of phage therapies are promising, as they reduce clinical symptoms or even bring full recovery. Because of the lack of comprehensive knowledge of the kinetics and immunomodulatory potential of bacteriophages, their use to eliminate pathogenic bacteria is still a developing field and research must be continued in order to provide a full understanding of their role in human and animal health.

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