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Effect of serum anti-phage activity on colibacillosis control by repeated phage therapy in broilers

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ABSTRACT

Anti-phage activity of serum is of importance in repeated phage therapy. Higher serum anti-phage activity has been associated with greater susceptibility of phages to neutralisation and phage therapy failure. In this study, *in vivo* and *in vitro* survivability and immunogenicity of four coliphages (TM1, TM2, TM3 and TM4) were investigated in naive chickens and chickens pre-immunised with phage TM1. Furthermore, two phages that displayed different survivability and immunogenicity (TM1 and TM3) were compared with respect to their efficacy in treating naive or pre-immunised (TM1) chickens suffering from colibacillosis. The efficacy of the treatments was evaluated based on body weight, relative organ weights, mortality, *E. coli* counts in the lungs as well as severity and frequency of internal organ lesions. At the end of the experiment, both naive and pre-immunised chickens treated with TM3 showed significantly lower mortality and higher body weights than untreated chickens and those treated with TM1. The same trend was observed in incidence and severity of organ lesions as well as relative spleen weight. However, naive chickens treated with TM1 also showed a shortened inflammation period as indicated by spleen weights. *E. coli* counts in the lungs of chicken treated with TM3 were lower than those of chickens treated with TM1 on days 3 and 10 post challenge. These data indicate that the outcome of phage therapy and the impact of serum anti-phage activity are highly phage-type dependent in broilers.

1. Introduction

Avian Pathogenic *Escherichia coli* (**APEC**) is among the greatest health threats to developed poultry industries (Collingwood et al., 2014). Disease, especially in broiler chickens, may be caused by a wide range of *E. coli* serotypes carrying various virulence factors (Ewers et al., 2004). APEC infections often result in colibacillosis with symptoms including fibrinous lesions of internal organs (airsacculitis, pericarditis, perihepatitis). In its most acute form, colibacillosis can lead to septicemia and sudden death (Ask et al., 2006; Collingwood et al., 2014). APEC infections lead to large financial losses due to high mortality, growth depression and condemnation rates (Mellata, 2013). Treatment of APEC infections usually involves antibiotic therapy, often at metaphylactic or flock levels, leading to further concerns over the emergence of more virulent or antibiotic resistant APEC strains (Talebiyan et al., 2014). The emergence of antibiotic resistance can lead to treatment failures, increased disease transmission and more severe outcome and heavier economic losses (Nhung et al., 2017).

Bacteriophages are viruses which infect and kill bacteria but have no known activity against eukaryotes. Phage therapy is the act of using bacteriophages as antibacterials in human or veterinary medicine and is one of the emerging methods with potential to overcome serious problems of drug-resistant bacteria (Ly-chatain, 2014). Many studies have been conducted on phage therapy in both human and veterinary medicine (Barrow et al., 1998; Biswas et al., 2002; Brussow, 2005; Kaźmierczak et al., 2014; Oduor et al., 2016; Kromann et al., 2017; Naghizadeh et al., 2019). Currently, however, only few studies examine the long-term use of phage therapy and the host immune response over time, and whether this response interferes with treatment efficacy in particular.

Bacteriophages are foreign to the host and, as such, may be subject to recognition and clearing by the host immune defence. Different groups have shown that under certain conditions, phages may be rapidly removed from circulation by the reticuloendothelial system of liver and spleen of the host (Dabrowska et al., 2014; Capparelli et al., 2007). Likewise, several groups have shown that the host may produce anti-phage-neutralising antibodies following exposure to phage therapy (Smith et al., 1987; Srivastava et al., 2004; Vitiello et al., 2005; Huff

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et al., 2010; Dabrowska et al., 2014; Hodyra-Stefaniak et al., 2015; Cisek et al., 2017). It has also been reported that the clearance rate of a phage by the immune system is substantially faster and stronger following a second phage exposure (Huff et al., 2010; Kim et al., 2008). However, it is important to consider that phages vary in their immunogenicity, and the intensity of host humoral immune response depends on the route of administration, dose of administered phages, the host immune status and the phage structure of proteins (Ceyssens et al., 2011; Lusiak-Szelachowska et al., 2014; Lusiak-Szelachowska et al., 2017; Żaczek et al., 2016).

Modification of phage surface structures could possibly affect host clearance rates (Vitiello et al., 2005; Kim et al., 2008). In a study examining the role of phage structural elements and host immune response, Vitiello et al. (2005) showed that minor variations in phage surface structures were sufficient to evade antibody binding and to enhance the effect of phage therapy. Similarly, Kim et al. (2008) found that chemical modification of the phages by non-immunogenic mPEG increased the half-life of circulating bacteriophages. Therefore, the ability of phages with different antigenic profiles to escape immune responses and to be resistant to neutralising antibodies could be anticipated and explored in a therapeutic setting.

To date, few studies have considered the effect of multiple phages administration on humoral immune response. To our knowledge, no study has investigated the anti-phage activity of serum (**AAS**) on the repeated phage therapy (**RPT**) outcome using multiple phages in an animal model. Therefore, the main objectives of our study were to: 1) compare *in vitro* and *in vivo* survivability of four distinct coliphages and the immunogenicity in either naive or phage pre-exposed chickens, and 2) use these results to identify phages with the lowest immunogenicity and measure the efficacy of phage therapy in commercial broilers with experimentally induced colibacillosis.

2. Material and methods

2.1. Bacterial strain and preparation

The *E. coli* O78:K80 strain, which was supplied by the Razi Vaccine and Serum Research Institute (Karaj, Iran), was used in this study. This isolate is a virulent strain and causes high mortality in chickens. The *E. coli* culture was prepared by inoculation of Luria-Bertani broth (**LB**) (Merck, Germany) and incubation at 37 °C with shaking for 3 h. The culture was subsequently removed from the incubator and kept at 4 °C. Bacterial concentrations were determined by making 10-fold serial dilutions of the culture and spread-plating the appropriate dilutions onto MacConkey agar (Merck, Germany) plates. Resulting colonies were enumerated after overnight incubation at 37 °C.

2.2. Bacteriophage isolation and amplification

A set of four heterogeneous bacteriophages designated vB_EcoS_TM1 (TM1), vB_EcoS_TM2 (TM2), vB_EcoS_TM3 (TM3) and vB_EcoS_TM4 (TM4) was used in these studies. The phages were originally isolated from wastewater samples obtained from poultry slaughterhouses and belonged to the family *Siphoviridae* in the order of *Caudovirales* as described previously (Naghizadeh et al., 2019). These phages have a broad host range against three strains of APEC [O1:K1, O2:K1, and O78:K80] and two strains of extra- intestinal pathogenic *E. coli* (ExPEC, [O126:K71 and O86:K61]) associated with human health. The phages were heterogeneous in morphologies, lytic patterns, the diameters of the plaques and restriction endonuclease digestion profiles (Naghizadeh et al., 2019). High titres of phages were prepared by a standard protocol of Merabishvili et al. (2009). Briefly, 50 µL of a 3-h culture of *E. coli* O78:K80 (OD600 = 0.6) was infected with 10^6–10^5 plaque forming units (**pfu**) of each bacteriophage in 4 mL of soft agar (0.7% agar in **LB**, 42–45 °C), overlaid onto plates containing 1.5% agar (Serva, Germany), incubated at 37 °C overnight. Following incubation, 300 µL of chloroform was added to each dish, and cultures were incubated at 4 °C for 1 h. Subsequently, 4 mL of phosphate-buffered solution (PBS) (0.14 *M* NaCl, 0.0027 *M* KCl, 0.01 *M*Na_2HPO_4, 0.0018 *M* KH_2PO_4; pH 7.4) was added to plates. The liquid and top agar layer was transferred to a sterile 14 mL tube and centrifuged for 20 min at 13,000 × g. The resulting supernatant was filter sterilised (0.22 µm membrane filter. Jet Biofil, China) to remove the residual bacteria. Phage titres were measured by routine double-layer plaque method in triplicate (Adams, 1959). Bacteriophages used in IgY and IgM ELISA assays were further purified by precipitation with 1 M NaCl and 10% polyethylene glycol (PEG) followed by centrifugation at 3000 rpm for 45 min. The precipitate was re-suspended in 0.05 M Tris−HCl buffer (containing 0.1 M NaCl and 10 mM $MgSO_4$, pH 7.5) and stored at −80 °C (Ahmadi et al., 2016).

2.3. Chickens and housing

One-day-old broiler chickens (Ross 308) were obtained from a local hatchery. Broilers were housed in a close-sided research facility under standard environmental conditions. All birds were fed the same commercial diets throughout the trial. Feed and water were available *ad libitum* throughout the entire experiment. All procedures described in this project were approved by the Animal Care and Use Committee and conducted in compliance with the Guidelines for Care and Use of Laboratory Animals at Tarbiat Modares University.

2.4. Phage survivability and immunogenicity

2.4.1. Immunisation of chickens

A previously described method (Hodyra-Stefaniak et al., 2015) with some modifications was used to raise phage antibodies in chickens. For the initial immunisation, one-day-old broiler chickens of the same weight were randomly divided into nine groups (N = 5) as shown in Fig. 1 and supplementary Table 1. Chickens were tested for the absence of phages. Chickens in groups 1–4 (pre-immunised) were administered 0.2 mL of phage TM1 filtrate (10^{11} pfu/mL) into the breast by i.m. injection on days 5 and 6. Chickens in groups 5–9 (naive) were injected with 0.2 mL of sterile PBS. Blood samples were collected from the wing vein on days 5 and 12 after the last injection and used as IgM and IgY-rich serum, respectively, to assess cross-neutralisation of the phages. Because our previous trials indicated a larger genome size and higher immunogenic property for phage TM1 compared to the other three phages, phage TM1 was selected for pre-immunisation.

2.4.2. Serum anti-phage activity in vitro

AAS was performed using the previously described method (Hodyra-Stefaniak et al., 2015) with minor modifications. In brief, serum obtained from pre-immunised chickens (by TM1 phage; 10^{11} pfu/mL as day-old) on days 5 (IgM rich serum) and 12 (IgYrich serum) after immunisation was used to assess AAS. Serum from sterile PBS-injected chickens (naive) was used as control. Each serum sample was applied as inactivated or non-inactivated serum (*i.e.* before or after inactivation of serum complement). Complement was inactivated by incubating each serum sample at 56 °C for 1 h. For AAS assessment, phages TM1, TM2, TM3 and TM4 (10^{11} pfu/mL) were individually mixed with the serum samples (1:1) and incubated at 37 °C for 2 h. Phage titres in the samples were determined by the double-layer plaque method as described above.

2.4.3. Phage circulation in naive and pre-immunised chickens

To assess AAS effect on circulation times of four phages, *in vivo* pre-immunised and naive chickens from groups 1 and 5, 2 and 6, 3 and 7, and 4 and 8 were injected with TM1, TM2, TM3 and TM4, respectively (see Fig. 1 and supplementary Table 1). Immediately after injection, 200 µL of blood was collected from wing veins of each chicken into a syringe with 10% EDTA in 10:1 ratio (V:V) and at different time points

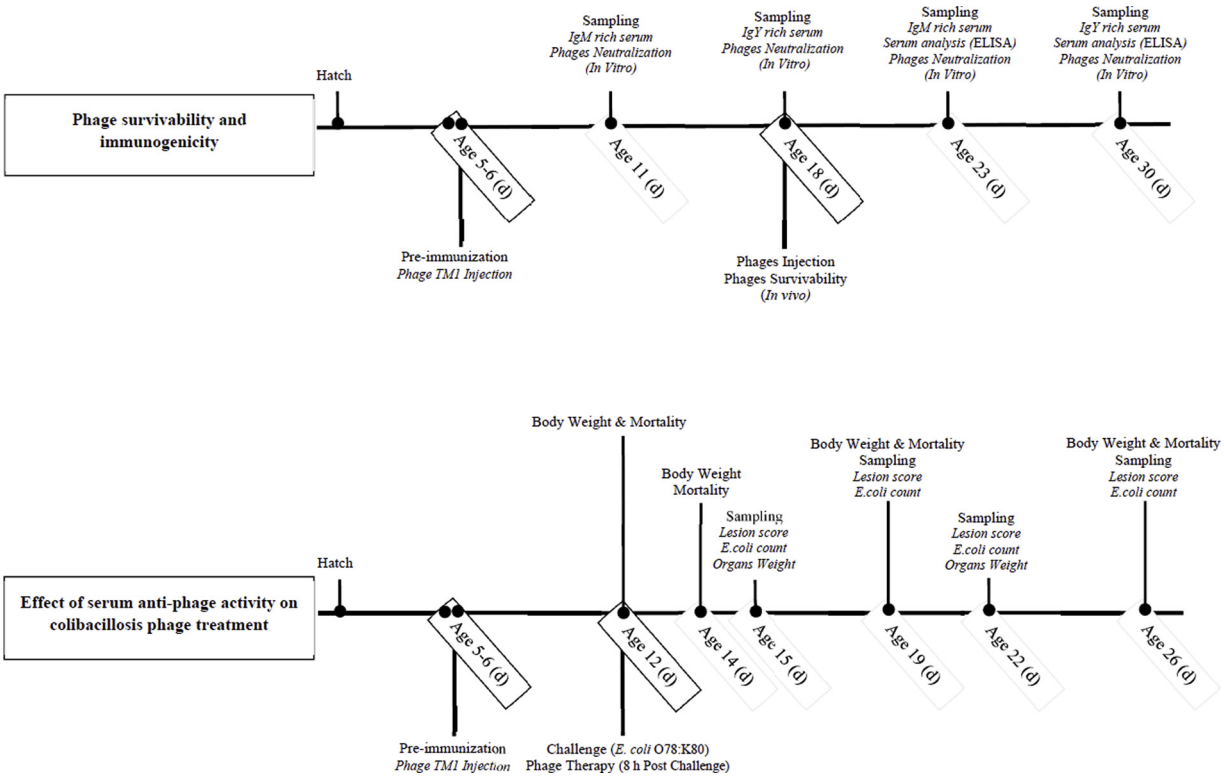

Fig. 1. Scheme of the studies.

during 24 h (see Fig. 2). Phage titres in the blood were detected by routine test dilutions on the surface of prepared host bacterial lawns. Five samples per group (N = 5) were tested, and the assay was repeated three times. After incubation, plaques were counted to determine phage titres. Serum samples were also taken from all chickens on days 5 and 12 after injection and stored at −20 °C until used for the enzyme-linked immunosorbent assay (ELISA).

2.4.4. Individual immunogenic properties of phages

Antigen-specific antibody titres to each phage in the serum of naive and pre-immunised chickens were determined by ELISA. In brief, flat-bottom, 96-well plates were coated (24 h) with each of the purified phages separately (TM1, TM2, TM3 and TM4), and blocking buffer (PBS with 1% BSA) was added. After 1 h, the plate was rinsed (3 times) with 250 µl of washing buffer (PBS with 0.1% BSA, pH 7.4). After incubation (90 min) with 100 µl of serum (1:400 dilution in PBS), the plate was rinsed (3 times) and incubated with 100 µl of horseradish peroxidase-conjugated, goat anti-chicken IgY or IgM (AA129 P, Serotec, Oxford, UK) diluted: 1:30,000 in PBS with 0.1% BSA. One hour later, the plate was rinsed (3 times), and 100 µl of TMB substrate (Clinical Science Product Inc, USA, cat. no. 01016-1-1000) was added. After 15 min, the reaction was stopped by the addition of 1 M H_2SO_4, and absorbance was measured at 450 nm. Anti-TM1 IgM and anti-TM1 IgY background levels were determined in chickens injected with PBS.

2.5. Effect of serum anti-phage activity on colibacillosis phage treatment

2.5.1. Experimental E. coli infection

The *in vivo* efficacy of RPT in controlling colibacillosis was studied by administration of either the same phage or a different phage in a second experiment. In total, 420 one-day-old broilers were randomly divided into seven groups with four replicate pens of 15 chickens each. Treatment groups are shown in Table 1: group I (naive-negative control), untreated, non-challenged chickens administered 0.2 mL of sterile PBS; group II (immunised-negative control), non-challenged chickens pre-immunised with 0.2 mL of phage TM1 (10^{11} pfu/mL) on days 5 and 6 of age; group III (positive control), chickens pre-immunised with phage TM1 on days 5 and 6 of age and challenged with *E. coli*; group IV (naive-TM1), naive chickens challenged with *E. coli* followed by 0.2 mL of phage TM1 (10^{11} pfu/mL); group V (immunised-TM1), chickens pre-immunised with phage TM1 on days 5 and 6 of age and challenged with *E. coli* followed by 0.2 mL of phage TM1 (10^{11}pfu /mL); group VI (naive-TM3), naive chickens challenged with *E. coli* followed by 0.2 mL of phage TM2 (1011 pfu /mL); group VII (immunised-TM3), chickens were pre-immunised with phage TM1 on days 5 and 6 of age and challenged with *E. coli* followed by 0.2 mL of phage TM2 (10^{11} pfu/mL).

Colibacillosis was induced at 12 d of age by injection of 0.2 mL (1×10^8 cfu mL^{-1}) of *E. coli* O78:K80 culture (grown in Luria-Bertani broth at 37 °C and shaken at 180 rpm; OD: 0.6) into the right thoracic airsac (between ribs 4 and 5). Phage therapy was administrated 8 h post challenge based on the results of a preliminary trial showing that *E. coli* O78:K80 had colonised in the lungs and spread into internal organs. Phage therapy was administered *via* i.m. injection of 0.2 mL (10^{11}pfu/mL) of each phage suspension into the breast.

The efficacy of the treatments was evaluated based on body weight (**BW**, measured weekly), mortality, and qualitative and quantitative determination of *E. coli* in the lungs and internal organs, respectively, and incidence and severity of the colibacillosis lesions. The chickens were weighed each week post challenge and at the end of the study. Total mortality was recorded manually as the average of the whole group from each experimental unit each week post challenge and at the end of the study.

On days 3, 7, 10 and 14 post challenge, eight chickens from each treatment group were euthanased by cervical dislocation and examined for incidence and severity of gross lesions, organ weights, and quantitative and qualitative isolation of *E. coli* from organs. After macroscopic examinations of the presence of tissue lesions or fibrinous exudates on the airsacs, heart and liver, the inner parts of heart, liver and spleen were aseptically swabbed onto the MacConkey agar (Merck, Germany). Afterwards, the lungs, liver, heart and spleen were excised and

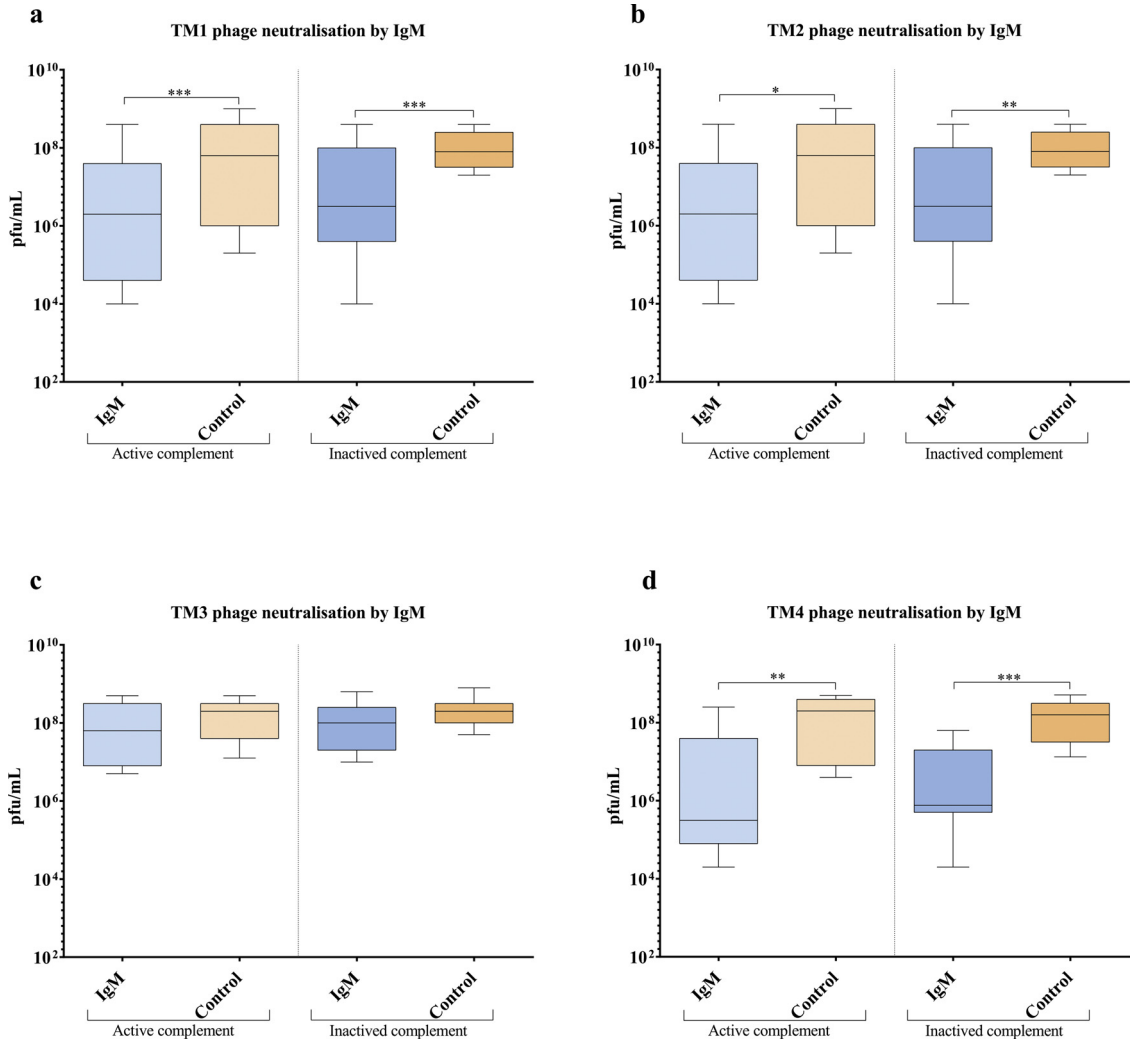

Fig. 2. Neutralisation of phages by IgM-rich serum. Serum concentration (pfu/mL) of phages TM1 (a), TM2 (b), TM3 (c) and TM4 (d) exposed to specific IgM-rich serum (pre-immunised) and control serum (naive). Chickens were pre-immunised i.m. with the TM1 phage (for dose scheme, see Materials and methods). Control chickens were injected with PBS. TM1-specific IgM-rich serum isolated from chickens on day 5 post TM1 immunisation (n = 5) was incubated for 2 h at 37 °C; phage activity was detected by plating. Serum complement was heat-inactivated by incubation at 56 °C for 1 h. Whiskers represent minimal and maximum values, boxes represent SD, and medians are marked by bars. Statistics: ANOVA *P < 0.05, **P < 0.005, ***P < 0.0005.

weighed. The weights of the organs were reported as the percentage relative to BW. The incidence and severity of lesions were evaluated individually and scored as described earlier (Naghizadeh et al., 2019) for all chickens that died during the experiment and for chickens sacrificed on day 28. Airsac thickness and the presence of tissue lesions or fibrinous exudates on the heart and liver were considered as

Table 1
Effect of RPT with either the same phage or distinct phage on BW (g) and mortality (%) of chickens[1].

Day post-challenge	BW						
	I	II	III	IV	V	VI	VII
0	293.92 ± 9.28	292.08 ± 6.96	290.00 ± 10.46	293.96 ± 8.48	293.42 ± 9.48	291.83 ± 9.31	291.75 ± 10.98
2	383.00 ± 15.36^{a}	384.25 ± 14.66^{a}	336.20 ± 7.73^{c}	357.55 ± 14.41^{bc}	339.00 ± 17.10^{c}	369.40 ± 12.37^{ab}	376.60 ± 12.67^{ab}
7	782.50 ± 42.76^{a}	808.75 ± 36.54^{ab}	563.58 ± 23.46^{e}	657.50 ± 26.83^{cd}	615.00 ± 42.80^{de}	730.12 ± 47.13^{bc}	727.50 ± 42.33^{bc}
14	1277.74 ± 27.91^{a}	1296.25 ± 28.07^{a}	827.50 ± 38.62^{e}	1041.27 ± 40.13^{c}	976.25 ± 55.38^{d}	1136.66 ± 51.69^{b}	1134.75 ± 46.15^{b}
Mortality	00.0 ± 0.0^{d}	00.0 ± 0.0^{d}	43. 3 ± 10.6^{a}	20 ± 3.6^{b}	36.6 ± 10.8^{a}	10 ± 3. 1^{c}	16.6 ± 5.9^{bc}

a–d: Values within a column followed by different superscripts are significantly different P < 0.0001.
The treatments were group I (naive-negative control), untreated, non-challenged chickens; group II (immunised-negative control), non-challenged chickens pre-immunised with 0.2 mL phage TM1; group III (positive control), pre-immunised chickens with phage TM1 challenged with *E. coli*; group IV(naive-TM1), naive chickens challenged with *E. coli*, followed by 0.2 mL phage TM1 (10^{11} pfu /mL); group V(immunised-TM1), pre-immunised chickens with phage TM1 challenged with *E. coli* followed by 0.2 mL phage TM1 (10^{11} pfu /mL); group VI (naive-TM3), naive chickens challenged with *E. coli* followed by 0.2 mL phage TM2 (10^{11} pfu /mL); group VII (immunised-TM3), pre-immunised chickens with phage TM1 challenged with *E. coli*, followed by 0.2 mL phage TM2 (10^{11} pfu /mL). Chicken were pre-immunised i.m. with TM1 phage (for dose schema, see Materials and methods). Control chickens were injected with sterile PBS.
[1] Values represent the mean body weights of survivors of four replicate pens of 15 chickens each.

airsacculitis, perihepatitis and pericarditis, respectively. The lungs of the birds were weighed, homogenised and subsequently serially diluted (10-fold) in PBS before plating on MacConkey agar (Merck, Germany) and incubating overnight at 37 °C. Pink colonies were considered as presumptive *E. coli* colonies and enumerated to determine the total concentration of *E. coli* (cfu/g) in the lungs. Total concentration of *E. coli* in the lung samples was compared across treatments to gauge the severity of colonisation (Lau et al., 2010).

2.6. Statistical analysis

Data of phage concentrations in the blood stream and serum were statistically evaluated using the Student's t-test. Standard box plots (box-and-whiskers plot) were used for graphical comparisons of the phage neutralisation by IgY and IgM antibodies. The test was used for comparison of two independent samples to determine differences between the pre-immunised and the naive groups.

The ELISA and the performance data (body weight and mortality) were compared among the groups using a two-way ANOVA. Fisher's exact tests were performed to determine significant differences between the treated and untreated *E. coli*-challenged groups and for qualitative analysis of *E. coli* from different organs and the severity of gross lesions. All statistical tests were performed in SAS (SAS Institute, 1999) or Prism 7.0C (Graph Pad Software Inc., La Jolla, CA). At P values of < 0.05, differences were considered significant.

3. Results

3.1. Phage survivability and immunogenicity

3.1.1. Phage TM3 displayed the longest in vitro survivability

Survivability of phages in the IgM-rich serum and IgY-rich serum was examined in order to assess AAS effect on stability of different phages *in vitro*. The infectivity of the phages in the IgM anti-TM1 and IgY anti-TM1 rich serums at 37 °C over 2 h was variable and depended on the phage (Figs. 2 and 3). Both IgM-rich serum and IgY-rich serum reduced the infectivity of all phages, except that of phage TM3 ($P < 0.05$; Figs. 2 and 3). Serum obtained from the TM1 pre-immunised chickens markedly decreased the infectivity of phage TM1 (Fig. 2a and Fig. 3b; $P < 0.0005$) as well as the infectivity of phage TM4 (Fig. 2d and Fig. 3d; $P < 0.0005$) compared to serum from naive chickens. However, this effect was observed to be lower on phage TM2 than on phage TM4 (5.2-log v. 6.2-log and 5-log v. 6-log, respectively). In contrast, phage TM3 infectivity in the pre-immunised serum was essentially equal to that in the naive serum (Fig. 2c and Fig. 3c). Moreover, the influence of serum complement on phage infectivity was not significant (Figs. 2 and 3).

3.1.2. Phage TM3 displayed the longest in vivo stability

Circulation times of four phages in pre-immunised chickens were compared to those in naive chickens in order to assess the AAS effect on stability of different phages *in vivo* (Fig. 4). In the pre-immunised chickens, the number of infective phage TM1 and phage TM4 decreased rapidly; no active phage was detected in the blood of chickens 4 h after injection, whereas phages were detectable in the naive chickens until 6 h after injection (Fig. 4a and d). In contrast, inactivation of phage TM2 in the pre-immunised chickens trended slightly slower; phage TM2 was detectable for 12 h after incubation (2–3 pfu/mL). Phage TM2 was detectable until 16 h (˜3–5 pfu/mL) after injection in the naive chickens (Fig. 4b). However, the longest circulating time in the bloodstream was observed for phage TM3, which was detected in concentrations of ˜10 pfus/mL after 24 h. Phage TM3 concentration in the bloodstream of the naive chickens ranged from 3-log/mL to 4-log/mL after 24 h after injection (Fig. 4c).

3.1.3. Immunogenicity varies between phages

IgM and IgY productions in response to the four phages were measured in naive and pre-immunised chickens in order to assess phage immunogenic properties. The primary response (IgM) and secondary response (IgG) to each phage in naive and pre-immunised chickens are shown in Fig. 5. IgM levels in the serum of naive chickens receiving phage TM1 and phage TM4 were significantly increased above background compared to the serum of those naive chickens receiving phage TM2 or TM3 ($P < 0.05$). A secondary response (IgY) was also induced by phage TM1 but not phage TM4 ($P < 0.005$). Comparative analysis of antibody levels in serum collected from the pre-immunised chickens on day 5 post phage injection revealed a significant increase in concentrations of anti-TM1 and anti-TM4 IgM in the pre-immunised chickens ($P < 0.005$). IgY was also induced by phage TM1 and phage TM4, and phage TM2 to a lesser extent ($P < 0.0005$). Phage TM3 was the least effective in stimulation of the immunological response, although IgY levels tended to be higher in the immunised chickens ($P > 0.05$).

3.2. Effect of serum anti-phage activity on colibacillosis phage treatment

3.2.1. Humoral response against TM1 does not affect efficacy of TM3 colibacillosis treatment

The effect of phage therapy using identical or dissimilar phage on BW and mortality of naive and pre-immunised chickens is shown in Table 1. No significant differences were observed in the BW of chickens on day 0 post challenge in the negative control groups (I and II) during the experimental period (Table 1). On day 2 post challenge, the lowest BW was observed in the positive control (group III) and in immunised-TM1 (group V, $P < 0.001$). On day 7 post challenge, the BW of naive-TM3 (group VI, 730 g) and immunised-TM3 (group VII, 727 g) was significantly higher ($P < 0.0002$) than the BW of naive-TM1 (group IV, 657 g) and immunised-TM1 (group V, 615 g). Similar results were observed in the BW on day 14 post challenge ($P < 0.0001$). However, the BW of chickens in immunised-TM1 (976 g) was significantly lower than the BW in naive-TM1 (1041 g) on day 14 post challenge. The lowest BW was observed in the positve control (828 g) on day 14 post challenge.

At the end of the experimental period, total mortality was 43.3% in the positive control (Table 1). Treatment of pre-immunised, *E. coli*-challenged chickens with phage TM3 significantly reduced mortality (10.3%) compared to the positive control (43.3%) and immunised-TM1 (36.6%, $P < 0.0001$). In addition, mortality of naive-TM3 chickens (10%) was significantly lower than the mortality of naive-TM1 chickens (20%, $P < 0.0001$). There was no mortality in any of the negative control groups during the experiment.

3.2.2. Naive and pre-immunised chickens treated with TM3 showed faster recovery from organ inflammation post challenge

The effect of the treatments on relative organs weight as an infection index is presented in Table 2. The sizes of the heart, liver and spleen were not significantly different among the negative control groups (I and II) throughout the experimental period ($P > 0.05$). In comparison with the negative control groups, enlargement of the hearts, lungs and livers of chickens in the positive control (group III) and immunised-TM1 (group V) was observed throughout the experimental period beginning from day 3 post challenge ($P < 0.05$). Additionally, significantly enlarged spleens of these chickens as well as chickens in immunised-TM1 were observed on days 3, 7, 10 and 14 post challenge ($P < 0.05$), while the sizes of the spleens in naive-TM1 (group IV), naive-TM3 (group VI) and immunised-TM3 (group VII) were not significantly different from those of chickens in the negative control groups on days 10 and 14 post challenge ($P > 0.05$). In immunised-TM1, enlarged organs were detected from days 3 to 14 post challenge, while enlargement of organs of chickens in immunised-TM3 was observed only on days 3 to 7 and 10 post challenge ($P < 0.05$). However, there were no significant differences in the relative organs

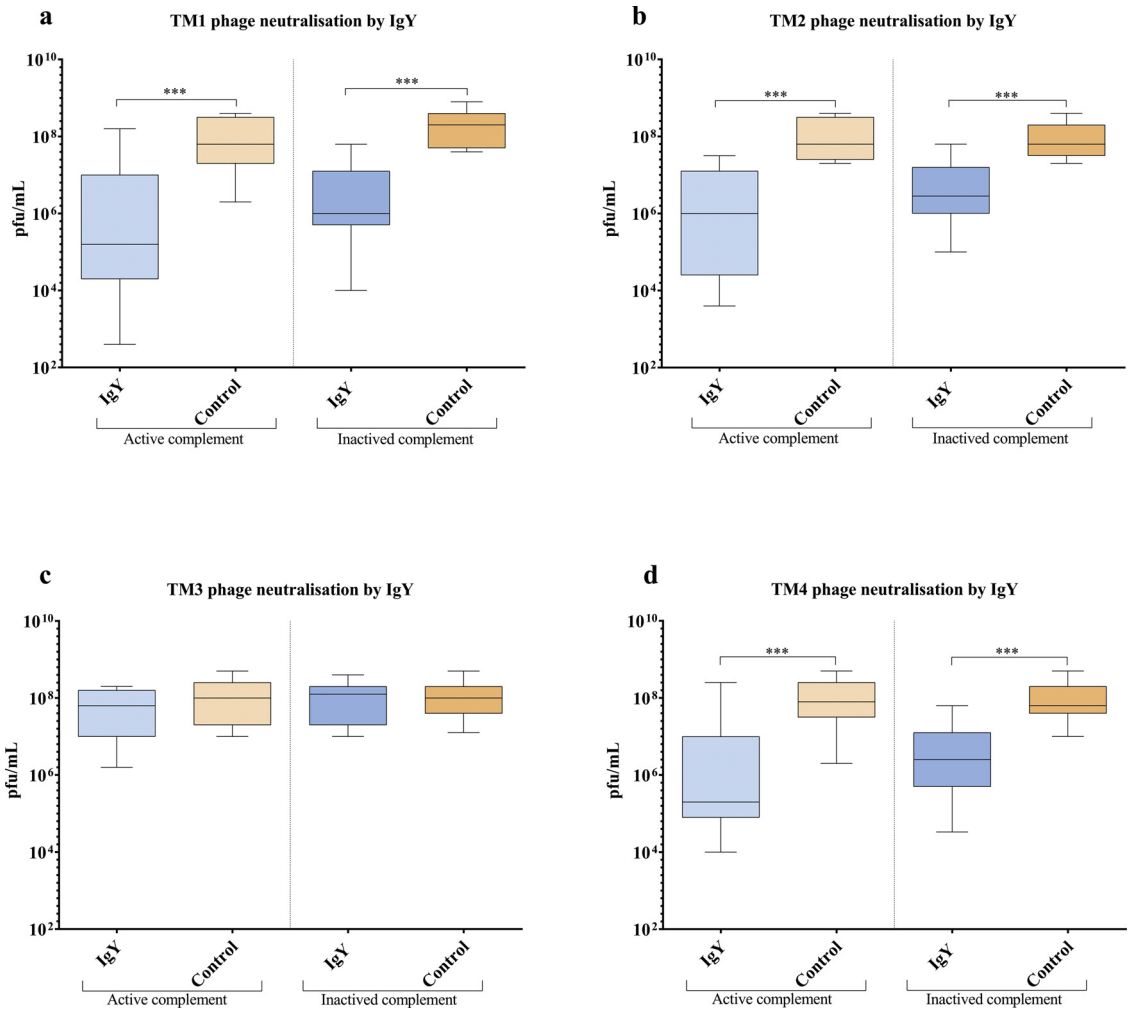

Fig. 3. Neutralisation of phages by IgY-rich serum. Serum concentration (pfu/mL) of phages TM1 (a) TM2 (b), TM3 (c) and TM4 (d) exposed to specific IgY-rich serum (pre-immunised) and control serum (naive). Chickens were pre-immunised i.m. with the TM1 phage (for dose scheme, see Materials and methods). Control chickens were injected with PBS. TM1-specific IgY-rich serum isolated from chickens on day 12 post TM1 immunisation (n = 5) was incubated for 2 h at 37 °C; phage activity was detected by plating. Serum complement was heat inactivated by incubation at 56 °C for 1 h. Whiskers represent minimal and maximum values, boxes represent SD, and medians are marked by bars. Statistics: ANOVA *P < 0.05, **P < 0.005, ***P < 0.0005.

weights of chickens in the positive control and immunised-TM1, except on day 14 post challenge when the lungs and liver relative weights of immunised-TM1 were significantly lower than that in the positive control (P < 0.0001). In addition, the relative liver and spleen weights of chickens in naive-TM1 or naive-TM3 were significantly lower than those in immunised-TM1 on day 14 post challenge (P < 0.0001). The largest sizes of the lungs, heart, liver and spleen were observed in *E. coli*-challenged groups throughout the experimental period (P < 0.05).

3.2.3. *TM3 therapy reduced gross lesions score and isolation of E. coli from different organs both in the naive and pre-immunised chickens*

The effect of the treatments on the frequency and severity of lesions is shown in Table 3 and Fig. 6. The severity of the lesions was highest in the positive control chickens (group III) and in immunised-TM1 (group V) throughout the experimental period (Table 3, P < 0.05). The incidence of airsac lesions on day 7 post challenge in naive-TM1 (group IV) or naive-TM3 (group VI) was significantly lower than that in the positive control (group III), in immunised-TM1 (group V) and immunised-TM3 (group VII) (P < 0.05). In addition, on day 7 post challenge, chickens in naive-TM3 showed the lowest incidence of pericarditis and perihepatits (P < 0.05). On days 7 and 10 post challenge, chickens in immunised-TM3 showed lower incidence and severity of pericarditis and perihepatits than chickens in immunised-TM1 (P < 0.05). Moreover, the incidence of pericarditis and perihepatits on day 10 post challenge in naive-TM1 chickens (2/8 and 1/8; P < 0.05), naive-TM3 chickens (1/8 and 0/8, respectively; P < 0.01) and immunised-TM3 chickens (2/8 and 1/8, respectively; P < 0.05) was significantly lower than the incidence in the positive control chickens (7/8 and 7/8, respectively) and immunised-TM1 chickens (6/8 and 6/8, respectively). On day 14 post challenge, the incidence of pericarditis and perihepatits was indifferent between *E. coli*-challenged groups, while the incidence of airsac lesions in groups naive-TM1, naive-TM3 and immunised-TM3 (1/8) was significantly lower than that in the positive control and immunised-TM1 (6/8 and 4/8, respectively; P < 0.05). In naive-TM3, perihepatitis occurred only on days 3 and 7 post challenge in 3/8 and 1/8 of the chickens, respectively, while in naive-TM1, perihepatitis could still be detected on day 10 post challenge (2/8 of the chickens).

The presence of *E. coli* in the heart, liver and spleen was assessed to determine *E. coli* dissemination in internal organs (Table 3). *E. coli* was recovered from the organs of chickens in the positive control and immunised-TM1 on days 3, 7, 10 and 14 post challenge; however, it was not recovered from the organs of the chickens in naive-TM3 and immunised-TM3 on days 7 and 10 post challenge, respectively. Furthermore, on day 7 post challenge, *E. coli* in the liver, heart and spleen of chickens in naive-TM3 was isolated only from 2/8 of the heart, 1/8 of

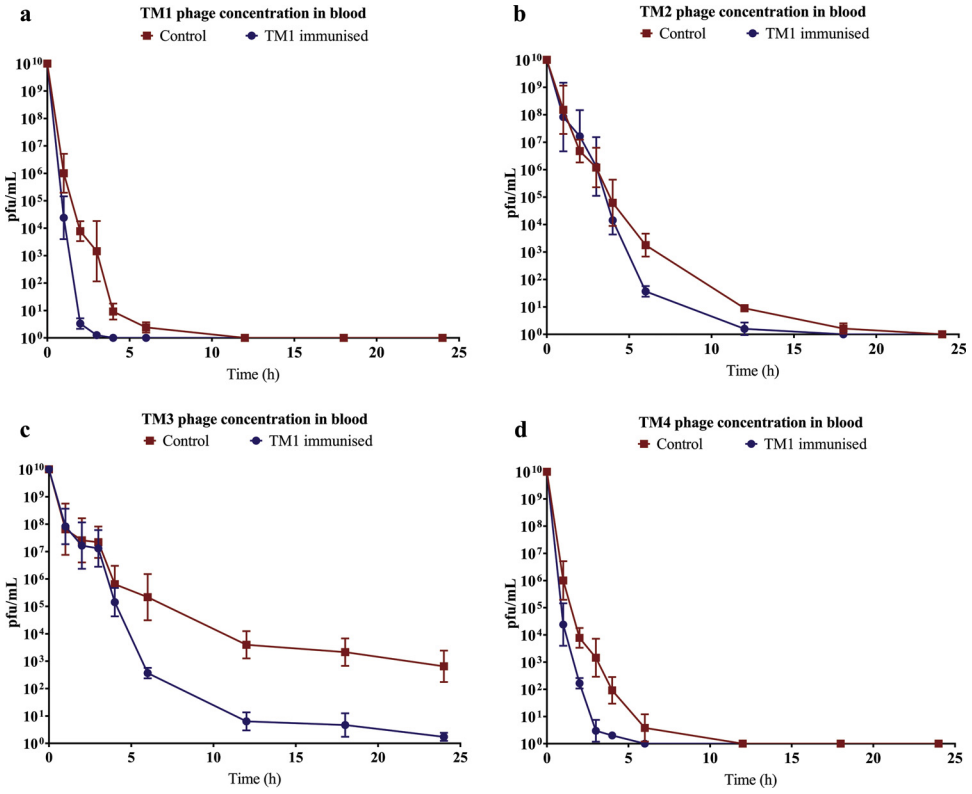

Fig. 4. Blood concentration (pfu/mL) of TM1 phage (a), TM2 phage (b), TM3 phage (c) and TM4 phage (d) of pre-immunised and control (naive) chickens. Chickens (n = 5) were pre-immunised i.m. with TM1 phage (for dose scheme, see Materials and methods). Control chickens were injected with PBS. All results are mean values ± 95% confidence interval (CI) for replicate sample preparations (n = 5), and mean values with no overlapping CI were interpreted as being statistically different.

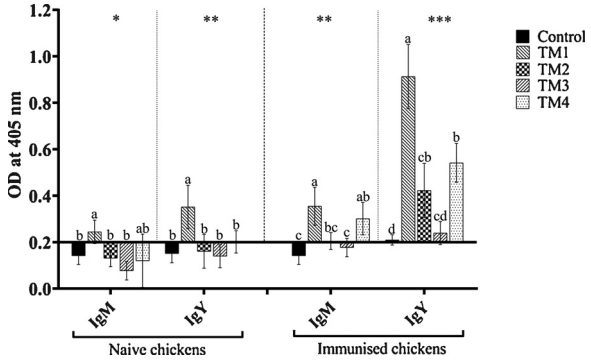

Fig. 5. Humoral response to individual phages in naive and pre-immunised chickens. Antibody levels were measured in the chicken serum on day 5 (IgM-rich) or day 12 (IgY-rich) after i.m. injection with TM1, TM2, TM3 and TM4 phages, respectively. Chicken were pre-immunised i.m. with TM1 phage (for dose scheme, see Materials and methods). Control chickens were injected with PBS, and TM1 antibody levels were measured in these chickens. The values represent the means of five replicate pens of one chicken per pen at each treatment. Statistics: ANOVA *P < 0.05, **P < 0.005, ***P < 0.0005.

the liver and 1/8 of the spleen, while in immunised-TM1, *E. coli* was still isolated on day 14 post challenge. No significant differences, however, were observed in the severity of lesions between naive-TM1, naive-TM3 and immunised-TM3 throughout the experimental period. Moreover, in all challenged groups, the severity of lesion decreased gradually from day 3 to day 14 post challenge.

3.2.4. TM3 therapy reduced the total number of E. coli in the lungs

The effect of treatments on total *E. coli* counts in the lungs is presented in Table 3. The number of *E. coli* in the lung samples was significantly reduced by ˜1.5 log10 cfu/g in immunised-TM3 chickens (group VII) compared to the positive control chickens (group III) and immunised-TM1 chickens (group V) on days 3, 7, 10 and 14 post challenge (Table 3). In the positive control, the concentration of *E. coli* was 7.39 log10 cfu/g on day 3 post challenge and decreased gradually to 5.07 log10 cfu/g on day 14 but remained significantly higher than that found in naive-TM1(group IV), naive-TM3, immunised-TM3 and in the negative control groups (I and II; P < 0.05). *E. coli* concentrations in naive-TM1 or naive-TM3 were increased by only ˜2 log10 cfu/g (from 3.21 to 5.24 and 3.21 to 4.99 log10 cfu/g, respectively) on day 3 post challenge, which was significantly different from the number of *E. coli* in the negative control groups (P < 0.05). Moreover, no significant differences were observed in the concentrations of *E. coli* between naive-TM3, naive-TM1 and immunised-TM3 throughout the experiment. Chickens in immunised-TM1 had significantly higher concentrations of *E. coli* in their lung samples compared to that found in immunised-TM3 (P < 0.05). The highest concentrations of *E. coli,* however, were observed in the positive control and immunised-TM1 throughout the experimental period (P < 0.05).

4. Discussion

Interference by anti-phage antibodies *in vivo* is considered to be among the most important limitations of RPT (Lusiak-Szelachowska et al., 2014; Sulakvelidze et al., 2001). Chemical modification of phages by non-immunogenic polymer monomethoxy-polyethylene glycol (mPEG) (Kim et al., 2008) or encapsulation in liposomes (Singla et al., 2016) has been proposed to shield them from antibody neutralisation hence improving RPT efficiency. The aim of this study was to identify phages with the lowest immunogenicity and measure the efficacy of RPT in commercial broilers with experimentally induced colibacillosis.

Phage numbers were quantified after incubation with IgM-rich serum and IgY-rich serum samples of all naive and pre-immunised chickens. Significant lower phage infectivity in the IgY-rich serum and IgM-rich serum was observed compared to serum from the naive chickens (control). The findings of our study are similar to those of Hodyra-Stefaniak et al. (2015) who found a significant inhibitory effect of IgG and IgM on survivability of active phage in the circulation and in numerous selected tissues. However, our result is in contrast to a study by Gorski et al. (2012) who reported that the induction of anti-phage

Table 2
Effect of RPT with either the same phage or distinct phage on relative weights of the lungs, heart, liver and spleen[1].

Relative weight[2]	Time post challenge (d)			
	3	7	10	14
Lungs (g/100 g BW)				
Group I	0.64 ± 0.15^{b}	0.69 ± 0.02^{c}	0.64 ± 0.03^{d}	0.56 ± 0.14^{b}
Group II	0.67 ± 0.11^{b}	0.69 ± 0.03^{c}	0.64 ± 0.07^{d}	0.55 ± 0.07^{b}
Group III	0.95 ± 0.15^{a}	1.12 ± 0.17^{a}	0.93 ± 0.14^{ab}	0.82 ± 0.19^{a}
Group IV	0.90 ± 0.15^{a}	0.97 ± 0.15^{ab}	0.78 ± 0.06^{c}	0.58 ± 0.05^{b}
Group V	0.86 ± 0.07^{a}	0.94 ± 0.06^{ab}	0.94 ± 0.11^{a}	0.79 ± 0.10^{a}
Group VI	0.86 ± 0.12^{a}	0.90 ± 0.12^{b}	0.82 ± 0.12^{bc}	0.48 ± 0.06^{b}
Group VII	1.03 ± 0.11^{a}	0.95 ± 0.12^{ab}	0.87 ± 0.06^{abc}	0.65 ± 0.08^{ab}
P-Value	0.0009	0.0008	< 0.0001	0.0025
Heart (g/100 g BW)				
Group I	0.63 ± 0.04^{b}	0.60 ± 0.11^{c}	0.60 ± 0.09^{b}	0.47 ± 0.03^{c}
Group II	0.58 ± 0.03^{b}	0.59 ± 0.05^{c}	0.53 ± 0.05^{b}	0.49 ± 0.05^{c}
Group III	0.96 ± 0.20^{a}	1.06 ± 0.05^{a}	1.02 ± 0.10^{a}	1.05 ± 0.17^{a}
Group IV	0.96 ± 0.22^{a}	1.02 ± 0.10^{ab}	0.87 ± 0.09^{b}	0.61 ± 0.12^{c}
Group V	0.89 ± 0.09^{a}	1.00 ± 0.12^{ab}	0.97 ± 0.14^{a}	0.87 ± 0.10^{b}
Group VI	0.82 ± 0.04^{a}	0.85 ± 0.13^{b}	0.96 ± 0.18^{a}	0.52 ± 0.08^{c}
Group VII	0.88 ± 0.09^{a}	0.92 ± 0.10^{ab}	0.98 ± 0.14^{a}	0.77 ± 0.09^{b}
P-Value	0.0018	< 0.0001	0.0003	< 0.0001
Liver (g/100 g BW)				
Group I	3.18 ± 0.61^{b}	3.58 ± 0.52^{c}	2.53 ± 0.13^{d}	2.52 ± 0.20^{c}
Group II	3.24 ± 0.46^{b}	3.65 ± 0.43^{c}	2.56 ± 0.23^{d}	2.65 ± 0.13^{c}
Group III	4.79 ± 1.45^{a}	6.47 ± 1.09^{a}	5.22 ± 1.07^{a}	4.43 ± 0.48^{a}
Group IV	4.64 ± 0.75^{a}	5.11 ± 1.08^{b}	3.96 ± 0.56^{bc}	2.95 ± 0.45^{bc}
Group V	4.93 ± 0.75^{a}	5.30 ± 0.31^{b}	5.02 ± 1.02^{ab}	3.43 ± 0.31^{b}
Group VI	4.41 ± 0.63^{ab}	5.22 ± 0.59^{b}	3.67 ± 0.33^{c}	2.65 ± 0.12^{c}
Group VII	4.92 ± 0.65^{a}	4.96 ± 0.25^{b}	4.35 ± 0.79^{abc}	2.81 ± 0.39^{c}
P-Value	0.0247	0.0003	0.0001	< 0.0001
Spleen (g/100 g BW)				
Group I	0.09 ± 0.02^{d}	0.08 ± 0.02^{c}	0.09 ± 0.02^{b}	0.10 ± 0.03^{b}
Group II	0.08 ± 0.02^{d}	0.10 ± 0.01^{c}	0.10 ± 0.01^{b}	0.10 ± 0.02^{b}
Group III	0.19 ± 0.05^{a}	0.20 ± 0.03^{a}	0.21 ± 0.05^{a}	0.19 ± 0.03^{a}
Group IV	0.16 ± 0.04^{abc}	0.15 ± 0.03^{b}	0.11 ± 0.02^{b}	0.11 ± 0.01^{b}
Group V	0.13 ± 0.01^{bdc}	0.21 ± 0.02^{a}	0.19 ± 0.03^{a}	0.17 ± 0.03^{a}
Group VI	0.12 ± 0.02^{dc}	0.14 ± 0.02^{b}	0.11 ± 0.01^{b}	0.10 ± 0.02^{b}
Group VII	0.18 ± 0.04^{ab}	0.14 ± 0.02^{b}	0.14 ± 0.05^{b}	0.13 ± 0.02^{b}
P-Value	0.0009	< 0.0001	0.0002	< 0.0001

a–c: Values within a column followed by different superscripts are significantly different.

[1] Values represent the mean ± SD body weights of survivors of four replicate pens of 15 chickens each.

[2] The treatments were group I (naive-negative control), untreated, non-challenged chickens; group II (immunised-negative control), non-challenged chickens pre-immunised with 0.2 mL phage TM1; group III (positive control), pre-immunised chickens with phage TM1 challenged with *E. coli*; group IV (naive-TM1), naive chickens challenged with *E. coli*, followed by 0.2 mL phage TM1 (10^{11} pfu /mL); group V (immunised-TM1), pre-immunised chickens with phage TM1 challenged with *E. coli* followed by 0.2 mL phage TM1 (10^{11}pfu /mL); group VI (naive-TM3), naive chickens challenged with *E. coli* followed by 0.2 mL phage TM2 (10^{11}pfu /mL); group VII (immunised-TM3), pre-immunised chickens with phage TM1 challenged with *E. coli*, followed by 0.2 mL phage TM2 (10^{11} pfu /mL). Chicken were pre-immunised i.m. with TM1 phage (for dose scheme, see Materials and methods). Control chickens were injected with sterile PBS.

antibodies does not necessarily relate to the loss of phage viability. The reason for this difference could be related to levels of antibodies where high levels of antibodies increase the risk of neutralisation. The levels of anti-phage antibodies depend on typical factors such as individual immunogenic characteristics of a phage and route of administration (Lusiak-Szelachowska et al., 2014).

Comparison of phage numbers in serum samples from chickens of naive and pre-immunised groups showed that the number of phages TM1, TM2 and TM4 was significantly lower for chickens of the pre-immunised groups compared to chickens of the naive groups (control; $P < 0.0005$). This difference suggests a cross-reaction due to homologies and structural similarities of the phages TM2 and TM4 with phage TM1 (Dabrowska et al., 2014). In this study, the pfu number of phage TM3 did not differ between the pre-immunised chickens and the naive chickens ($P > 0.05$). The differential survivability of the phages in serum and the variation in cross-reaction of phages were confirmed by quantifying the phage numbers circulating in the blood stream of individual chickens (Figs. 2, 3 and 4). Intriguingly, a similar ranking was observed for peripheral counts of phages in both naive and pre-immunised chickens. Of notice, the number of phage TM3 in the circulation blood was not as stable as in the serum, indicating the presence of alternative clearance mechanisms of the innate immune response.

Although complement and opsonins as key elements of the innate immune system play contributory roles in bacterial clearance, in our study similar to a study by Hodyra-Stefaniak et al. (2015), no significant effect of the complement system was found on the phage activities in either the naive or pre-immunised chickens' serum after 2 h of incubation. The influence of the complement system in mice, however, was found to have significant effect on phage activities (Dabrowska et al., 2014). Complement activation involves a complex enzymatic cascade which can be influenced by many factors, including how blood is collected and processed, the extent of the dilution of serum, and the time and temperature of incubation (Xu et al., 2008). These factors may help to explain why there is considerable dissimilarity among previous studies on the phage inactivation by the complement system. However, more work is needed to elucidate the importance of innate immune system (*e.g.* phagocytic cells in phage clearance).

The specific anti-phage antibodies may bind to phage proteins that are essential for infecting the host bacteria and thereby resulting in loss of phage antibacterial activity (Dabrowska et al., 2014). The cross-reactions with specific antibodies are strongly dependent on the level of structural similarity of the phage antigens (Dabrowska et al., 2014; Hodyra-Stefaniak et al., 2015).

Although the structural protein differences between the phages were not addressed in this study, considering the effect of phage structural elements on phage therapy results (Vitiello et al., 2005; Kim et al., 2008), it can be hypothesised that variant structural proteins of phage TM3 may explain the differential survivability of phage TM3 in the bloodstream and in the serum of pre-immunised chickens. This was also supported by our ELISA results in which the level of antibodies against phage TM3 in the chickens was similar to that of naive chickens, whereas significantly elevated levels of IgY and IgM antibodies against phages TM2 and TM4 were detected in the pre-immunised chickens (Fig. 3). Therefore, low antibody production in response to phage TM3 administration found in naive and pre-immunised chickens may be related to its low immunogenicity. It has been reported that differentiated capsid elements of phages are responsible for differences in their antigenicity and immunogenicity (Lusiak-Szelachowska et al., 2014; Prisco and de Berardinis, 2012; Vitiello et al., 2005).

Since the number of phages in body fluids may influence the availability of phages to the site of infection and their therapeutic efficacy, we also performed an animal experiment applying a disease model and extended these investigations beyond TM3 and TM1 for their ability to treat colibacillosis in chickens. In this study, BW of chickens in all *E. coli*-challenged groups was significantly lower than unchallenged groups. We found that the adverse impact of *E. coli* challenge on BW was reduced by phage treatment, with a more pronounced effect with the phage TM3 treatment *versus* TM1 phage treatment in either the pre-immunised or the naive chickens. Importantly, mortality as an essential outcome variable to evaluate the efficacy of a treatment, was significantly lower in the chickens treated with phage TM3 than in those treated with phage TM1. Similar findings have previously shown that phage therapy reduces colibacillosis disease symptoms in terms of body weight loss, mobility and mortality in chickens challenged with *E. coli* (Naghizadeh et al., 2019; Kaikabo et al., 2017; Zhao et al., 2017; Oliveira et al., 2010). Our result also confirms previous studies (Huff

Table 3
Effect of RPT with either the same phage or different phage on the frequency and mean severity of lesions, presence of *E. coli* in the heart, liver and spleen, and the total number of *E. coli* in the lungs.

Treatments[1]	Time post challenge (3 d)[2]							
	Birds with lesion			*E. coli* isolation[3]			Mean lesion scores[4]	*E. coli* counts (Log10 cfu/g)
	AS	H	L	H	L	S		L
Group I	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{d}	3.36 ± 0.15^{d}
Group II	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{d}	3.29 ± 0.17^{d}
Group III	8/8	5/8	6/8	5/8	5/8	4/8	3.33 ± 1.36^{a}	7.39 ± 0.46^{a}
Group IV	7/8	4/8	3/8	3/8	3/8	3/8	2.16 ± 1.47^{bc}	5.61 ± 0.33^{c}
Group V	8/8	5/8	3/8	5/8	4/8	5/8	2.83 ± 1.47^{ab}	6.67 ± 0.17^{b}
Group VI	5/8	3/8	3/8	3/8	2/8	3/8	1.16 ± 0.75^{c}	5.45 ± 0.38^{c}
Group VII	7/8	3/8	4/8	4/8	3/8	2/8	1.66 ± 0.52^{bc}	5.75 ± 0.26^{c}
Treatments[1]	Time post challenge (7 d)[2]							
	Birds with lesion			*E. coli* isolation[3]			Mean lesion scores[4]	*E. coli* counts (Log10 cfu/g)
	AS	H	L	H	L	S		L
Group I	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{c}	3.21 ± 0.22^{c}
Group II	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{c}	3.54 ± 0.24^{c}
Group III	8/8	7/8	7/8	6/8	5/8	4/8	4.33 ± 1.03^{a}	6.92 ± 0.54^{a}
Group IV	3/8*	3/8	3/8	3/8	3/8	2/8	1.83 ± 0.98^{b}	5.24 ± 0.50^{b}
Group V	8/8	7/8	7/8	5/8	4/8	4/8	3.16 ± 1.94^{a}	6.66 ± 0.71^{a}
Group VI	3/8*	2/8*	1/8*	2/8	1/8	0/8	0.66 ± 0.82^{b}	4.99 ± 0.72^{b}
Group VII	4/8	4/8	2/8	3/8	1/8	2/8	1.17 ± 1.7^{b}	4.61 ± 0.27^{b}
Treatments[1]	Time post challenge (10 d)[2]							
	Birds with lesion			*E.coli* isolation[3]			Mean lesion scores[4]	*E. coli* counts (Log10 cfu/g)
	AS	H	L	H	L	S		L
Group I	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{c}	4.17 ± 0.20^{b}
Group II	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{c}	4.11 ± 0.17^{b}
Group III	7/8	7/8	6/8	4/8	5/8	4/8	2.17 ± 1.94^{a}	5.36 ± 0.92^{a}
Group IV	3/8	2/8*	1/8*	2/8	1/8	0/8	0.83 ± 0.98^{bc}	4.61 ± 0.30^{b}
Group V	7/8	6/8	6/8	5/8	4/8	3/8	2.00 ± 1.67^{ab}	5.48 ± 0.39^{a}
Group VI	2/8*	1/8***	0/8***	0/8	0/8*	0/8	0.16 ± 0.41^{c}	4.10 ± 0.32^{b}
Group VII	3/8	2/8*	1/8*	0/8	0/8	0/8	0.50 ± 0.84^{c}	4.36 ± 0.31^{b}
Treatments[1]	Time post challenge (14 d)[2]							
	Birds with lesion			*E.coli* isolation[3]			Mean lesion scores[4]	*E. coli* counts (Log10 cfu/g)
	AS	H	L	H	L	S		L
Group I	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{c}	4.37 ± 0.35^{b}
Group II	0/8	0/8	0/8	0/8	0/8	0/8	0 ± 0.0^{c}	4.41 ± 0.25^{b}
Group III	6/8	4/8	4/8	4/8	3/8	3/8	1.83 ± 2.14^{a}	5.07 ± 0.47^{a}
Group IV	1/8*	0/8	0/8	1/8	0/8	1/8	0.17 ± 0.40^{b}	4.37 ± 0.43^{b}
Group V	4/8	4/8	3/8	3/8	1/8	2/8	1.00 ± 1.55^{ab}	4.74 ± 0.52^{ab}
Group VI	1/8*	0/8	0/8	0/8	0/8	0/8	0.50 ± 0.55^{b}	4.15 ± 0.12^{b}
Group VII	1/8*	0/8	0/8	0/8	0/8	0/8	0.50 ± 0.55^{b}	4.26 ± 0.16^{b}

Lesion score: Airsac, (0) normal; (1) no lesions; (2) slight lesions (mild cloudiness of airsacs with mild fibrinous exudate); (3) thickened airsac membranes; (4) meaty appearance of airsacs. Heart and pericardium: (0) normal; (1) turbid with excessive or cloudy fluid in the pericardial; (2) acute pericarditis. Liver: (0) normal; (1) slight amounts of fibrinous; (2) marked perihepatits.

[1] The treatments were group I (naive-negative control), untreated, non-challenged chickens; group II (immunised-negative control), non-challenged chickens pre-immunised with 0.2 mL phage TM1; group III (positive control), pre-immunised chickens with phage TM1 challenged with *E. coli*; group IV (naive-TM1), naive chickens challenged with *E. coli*, followed by 0.2 mL phage TM1 (10^{11} pfu /mL); group V (immunised-TM1), pre-immunised chickens with phage TM1 challenged with *E. coli* followed by 0.2 mL phage TM1 (10^{11}pfu /mL); group VI (naive-TM3), naive chickens challenged with *E. coli* followed by 0.2 mL phage TM2 (10^{11}pfu /mL); group VII (immunised-TM3), pre-immunised chickens with phage TM1 challenged with *E. coli*, followed by 0.2 mL phage TM2 (10^{11} pfu /mL). Chicken were pre-immunised i.m. with TM1 phage (for dose scheme, see Materials and methods). Control chickens were injected with sterile PBS.

[2] The number of samples positive with signs. The number of chickens investigated are displayed; the comparison between groups III, IV, V, VI and VII was performed using Fisher's exact test. *P < 0.05, **P < 0.01 and ***P < 0.001.

[3] AS = airsacs; H = heart; L = liver; S = spleen.

[4] Combined lesion scoring values for severity of aerosacculitis, pericarditis and perihepatitis.

et al., 2010; Lusiak-Szelachowska et al., 2014) reporting that prior exposure of animals to the same phage type reduced the antibacterial efficacy of the phage. Here, a higher therapeutic efficacy of the TM3 phage-treated chickens compared to those treated with phage TM1 could be attributed to the resistance of phage TM3 to inactivation by anti-TM1 phage antibodies in the pre-immunised chickens.

Although phages TM1 and TM3 both had a broad host range, especially against *E. coli* O78:K80 in *in vitro* when administered individually in quails (Naghizadeh et al., 2019), we noted they differed in their abilities to treat colibacillosis. The naive chickens challenged with *E. coli* and treated with phage TM1 had a significantly lower body weight and higher mortality rate (Table 1) compared to those treated with phage TM3. The mechanism for this is unknown, but a possible explanation for these differences might be the intrinsically variant suitability of the phages to neutralisation (Ceyssens et al., 2011) as well as variant sensitivity of each phage to endogenous proteases (Kim et al., 2008). These intrinsic characteristics of phages, therefore, may affect the number of phages *in situ* and their therapeutic effectiveness (Huff et al., 2006). This may explain why there is considerable dissimilarity among previous studies on the phage therapy outcome. However, more detailed studies are needed to compare *in vivo* phages' therapeutic effectiveness.

In response to an infection, the host immune system often generates pro-inflammatory responses mediated by the release of cytokines and

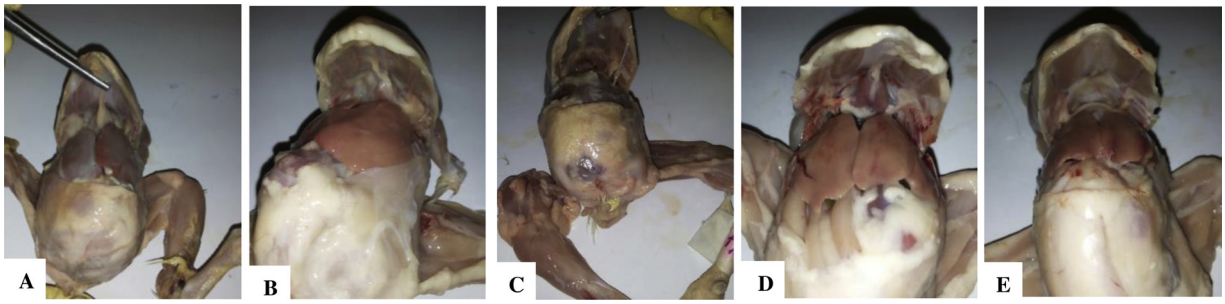

Fig. 6. Pictures of the severity of colibacillosis lesions in groups of chickens challenged with *E. coli*. (A) Pre-immunised chickens challenged with *E. coli* on day 12 of age and not treated with phage (Group III); (B) naive chickens challenged with *E. coli* and treated with phage TM1 on day 12 of age (Group IV); (C) Pre-immunised chickens challenged with *E. coli* and immediately treated with TM1 phage on day 12 of age (group V); (D) Naive chickens challenged with *E. coli* and treated with phage TM3 on day 12 of age (Group VI); (E) Pre-immunised chickens challenged with *E. coli* and treated with phage TM3 on day 12 of age (Group VII).

chemokines from macrophages, neutrophils and other immune cells in infected tissues as a consequence of the recognition of the pathogens (Wang et al., 2015). These events may lead to the accumulation of plasma proteins in the infected organs where it appears as swelling/edemas (Wang et al., 2015). Therefore, the significantly higher relative spleen and liver weights of *E. coli*-challenged chickens treated with phage TM1 on days 10 and 14 post challenge compared to those treated with phage TM3 are likely to reflect inflammation and may be indicative of the severity of the infection in those chickens (Table 2). Moreover, the frequency of pericarditis and perihepatitis, which indicate systemic bacterial infection (Kemmett et al., 2014), was also lower in phage TM3-treated, *E. coli*-challenged chickens compared with those treated with phage TM1 or with untreated chickens (Table 2). This result indicates the higher efficacy of therapy by phage TM3 compared to phage TM1. Our results also showed that the effectiveness of a specific anti-phage antibody to bind to a phage and to remove it from the body fluid may not necessarily affect other phages.

In conclusion, we believe that different phage types might react differently to neutralisation by antibodies. Therefore, the conclusions drawn from inactivation of a particular phage with specific phage antisera cannot be extrapolated to other phage types. The results strongly indicate that screening of phages for their capability to avoid antibody neutralisation may provide an important tool to achieve the maximum effectiveness of RPT.

Disclosure statement

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.018.

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