In vivo toxicity study of phage lysate in chickens

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SHORT COMMUNICATION

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Abstract 1. Bacteriophage (phage) crude lysate of Gram-negative bacteria often contains bacterial debris, including lipopolysaccharides found in the outer membrane of the cell wall, which are potentially toxic.
2. In this study, an *in vivo* evaluation of the toxicity of a suspension of three phages to control pathogenic *Escherichia coli* strains in poultry was performed.
3. Eighteen commercial layers, 7 weeks old, were intramuscularly injected with phage lysate (8.21×10^4 Endotoxin Units/dose). The control group was injected with sterile Luria Bertani (LB) broth.
4. Bird prostration and decrease in body weight gain and water intake per gram of body weight were observed only on the day of the inoculation in the challenged group. Over the following 6 d, no differences were observed in the chickens' activity.
5. These results support the view that phage crude lysate carrying endotoxins are not toxic for chickens.

INTRODUCTION

Lytic bacteriophages (phages) are viruses that infect and promote bacterial lysis through a multiple-step process: they multiply in the host bacteria and lyse it at the end of the cycle, after immediate replication of new phage particles. They are regarded as good candidates for antimicrobial therapy, as they provide an opportunity to control bacterial infections. Bacteriophages are safe once they exclusively infect bacterial cells, having no activity against animal or plant cells. Like all viruses, phages are metabolically inert in extracellular form and they are ubiquitous in nature (Huff *et al.,* 2005). Nevertheless, as phage infections culminate in lysis of bacteria, there is a consequent release of cell wall components to the environment as cell debris. In this process, Gram-negative bacteria release endotoxin, whose biological activity is associated with complexes of lipopolysaccharides

(LPS), present in the outer layer membrane. This can lead to undesired side effects with phage therapy. The LPS toxicity is associated with the lipidic component of the molecule, known as "Lipid A", while the immunogenicity is associated with the polysaccharide component, known as "O-specific side chain" or "O-antigen" (Culbertson and Osburn, 1980; Prins *et al.*, 1994; Williams, 2001*b*; Todar, 2002; Boratyński *et al.*, 2004). Thus, LPS are present in the cellular debris in crude phage lysates, easily passing through filters used to remove whole bacteria from phage suspensions (Williams, 2001*b*).

The endotoxins induce a variety of inflammatory responses, being often part of the pathology of Gram-negative bacterial infections. Although animals vary in their susceptibility to endotoxins, the sequence of pathophysiological reactions follows a general pattern: a latent period followed by physiological distress. Immunologic and neurological system activation,

induction of blood coagulation, general metabolic effects, alteration of blood cell populations, pyrogenicity, production of endotoxic shock and hepatotoxicity are some of the known reactions to an endotoxin parenteral challenge, promoting symptoms like fever, diarrhoea, prostration and, in many cases, shock and death (Culbertson and Osburn, 1980; Williams, 2001a). Removal of endotoxins from solutions intended to be used in humans or animals is therefore an important area for study, but the success of this procedure is dependent on the initial composition of the mixture (Petsch and Anspach, 2000; Boratyński et al., 2004). Ultrafiltration and size-exclusion chromatography should theoretically provide a way of separating components differing in molecular mass. However, the application of these two down stream processes in the purification of phages removes only some of the contaminants present in the phage suspension. Despite their relatively low molecular weight (4–20 kDa for LPS monomer), endotoxins are not effectively removed as they tend to aggregate forming structures similar to micelles and vesicles, ranging in molecular weight from 300 000 to 1 million, with diameters up to 0·1 μm (Williams, 2001b; Boratyński et al., 2004). Other approaches to achieving destruction or removal of endotoxins, like hydrolysis with acid or base, oxidation, alkylation, heat treatment and treatment with polymicin B have been reported (Davies, 1999). However, the economical viability of scaling up the process and the possibility of compromising the recovery rate of the phages needs evaluation. Petsch and Anspach (2000) report that the question of economical endotoxin removal has occupied many investigators and has been the reason for process rearrangements.

Our in vivo trial with an E. coli phage crude lysate, administered intramuscularly to chickens, was conducted in order to evaluate the endotoxin effect and to assess the importance of endotoxin removal in ensuring the safety of this phage product for the target species.

MATERIALS AND METHODS

E. coli phage lysate

Crude phage suspensions were prepared by inoculating a single phage plaque of the phages phi F78E, phi F258E and phi F61E in 10 ml of the respective E. coli host H561E, H816E and H161E, mid-log grown (3–4 h) into Luria Bertani (LB) broth (Sigma) (Oliveira et al., 2009). This was followed by overnight incubation at 37°C with shaking (120 rpm). The suspension was centrifuged (9000 × g) for 10 min (rotor 19 776, Sigma 3-16k), filtered through a 0·22-μm membrane (Millipore) and, following the same procedure as previously, inoculated again in 100 ml of mid-log grown culture of the respective E. coli host. Incubation was performed at 37°C with shaking (120 rpm), centrifugation was at 9000 × g for 10 min and filtration was through 0·22 μm. The phage crude lysate was stored at 4°C. The number of phages in this suspension was determined as described by Adams (1959) with minor modifications. Successive dilutions of the phage suspension were performed in phage buffer (100 mmol/l NaCl (Sigma), 8 mmol/l MgSO₄ (Sigma), 50 mmol/l Tris (Sigma), pH 7·5) and 100 μl of each dilution together with 100 μl of the respective bacteria host suspension were mixed with 3 ml of LB 0·6% top agar layer and placed over a 1·5% LB agar bottom layer. Plates were incubated at 37°C for 12 h and phages enumerated in the higher dilution with distinct plaques. Phage titration was performed in triplicate.

Measurement of endotoxin concentration

The concentration of LPS present in the E. coli phage lysate was measured using the Limulus Amebocyte Lysate assay (LAL) (Bio-Whittaker), which is based on the activation of Limulus Lysate by endotoxins (Prins et al., 1994). The procedure was as instructed by the supplier, using a spectrophotometer (Bio-TEK Synergy HT). This method was approved by FDA for detection and quantification of endotoxins (FDA, 1985).

Experimental design

This study was conducted according to the Federation of European Laboratory Animal Science Associations (FELASA) principles of animal welfare, and the experiment was designed in accordance to the European Council Directive of 24 November 1986 (86/609/EEC) guidelines, on the approximation of laws, regulations and administrative provisions of the member States regarding the protection of animals used for experimental and other scientific purposes.

Thirty-six healthy 7-week-old growers (Rhode Island Red) were used. Two groups of 18 chickens were selected at random and housed, three per cage, in a temperature and relative humidity controlled animal room, with a 12 h L:12 h D cycle. Birds were individually identified by leg rings. Feed and water were provided ad libitum. A volume of 1 ml of the previously prepared phage suspension and 1 ml of sterile LB broth were injected intramuscularly, respectively in the challenged group (CHG) and in the control group (CG), only on the first day. The chickens' body weight (BW) was recorded the day before and every day after challenge.
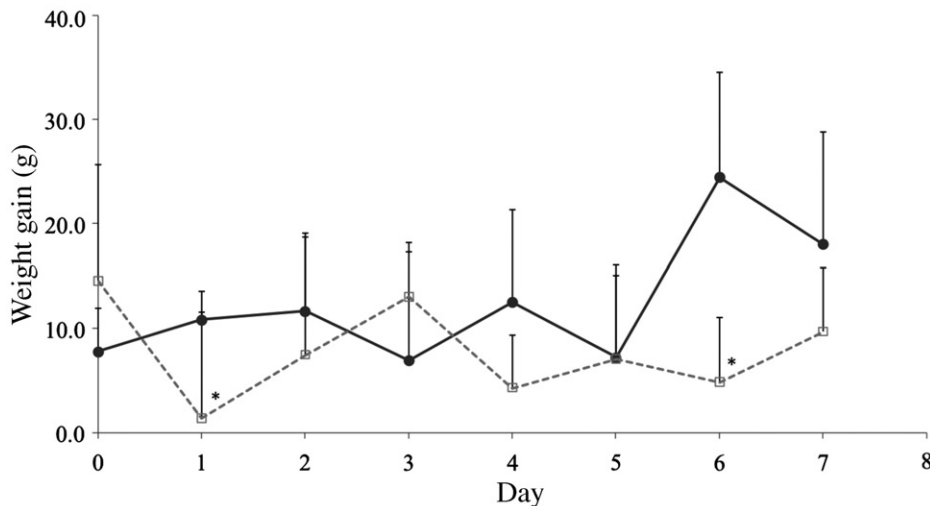
Figure 1. *Chickens' daily BW gain. In the figures, the solid line represents CG variation in body weight gain and the dashed line represents the CHG variation. Error bars represent standard deviations of experimental data from the 6 cages of three animals each. *Statistically different from CG.*

In order to avoid unnecessary discomfort to the animals, the evaluation of chickens' reaction to challenge was based on behavioural observations. Healthy chickens were recognised by their standing position, with neck strong and straight, flat feathers against the body and high frequency of seeking food and water. In contrast, if chickens were sitting, lying still, with the neck weak and drawn in, raised feathers, and not seeking food or water, they were reported as prostrate. Food and water consumption were recorded daily. On day 7, all chickens were humanely killed by isofluorane (IsoFlo®, Abbott) inhalation and submitted to *post mortem* examination.

Statistical analysis

Statistical analysis was undertaken for all dependent variables: BW gain, feed and water intake per gram of BW. CHG means were compared with CG means at each data collecting period. Statistical variance analysis was performed using Kruskal–Wallis test in SPSS v15·00 software. Statistical significance was defined as $P = 0.05$.

RESULTS

E. coli phage lysate

The *E. coli* phage lysate is a mixture of three phages phi F78E, phi F258E and phi F61E, with the concentration of each phage being 1.67×10^8 pfu/ml, 2.5×10^8 pfu/ml and 3.0×10^8 pfu/ml, respectively. The LAL test revealed that the LPS concentration present in this suspension was 8.21×10^4 Endotoxin Units (EU)/ml.

In vivo challenge with phage lysate

A volume of 1 ml of phage lysate was administered intramuscularly to the chickens of the CHG, with the total amount of LPS being, on average, 2.32×10^5 EU/kg BW.

During the *in vivo* experiment, bird prostration was observed only on the day of the inoculation (day 1) in CHG. One bird died one hour after inoculation but *post mortem* analysis detected no macroscopic lesions in the internal organs. During the following 6 d there were no visual differences in activity between the two groups. Regarding BW gain (Figure 1), on day 1 and day 6, this variable decreased in CHG birds ($P = 0.043$ and $P = 0.010$, respectively). Feed and water intake per gram of BW did not diverge significantly between groups (Figures 2 and 3). Water intake per gram of BW at day 1 decreased in CHG birds, but with no relevant differences between groups ($P = 0.065$). On day 4, there was a decrease ($P = 0.035$) in CHG, however this tendency was no longer observed in the following days.

DISCUSSION

Phage therapy has been considered an important alternative to the administration of antibiotics in the treatment of severe *E. coli* infections in birds (Huff *et al.*, 2004, 2005). One concern is the presence of endotoxins in the phage crude lysate (Projan, 2004). In the present work, 8.21×10^4 EU/ml LPS were found in the prepared phage cocktail used. Nevertheless, the chickens were challenged with 1 ml of this phage lysate containing approximately 2.32×10^5 EU/kg BW, which was not supposed to be lethal, since, and according to Culbertson and

Figure 2. *Chickens' feed consumption per gram of BW. In the figures, the solid line represents CG variation in body weight gain and the dashed line represents the CHG variation. Error bars represent standard deviations of experimental data from the 6 cages of three animals each.*
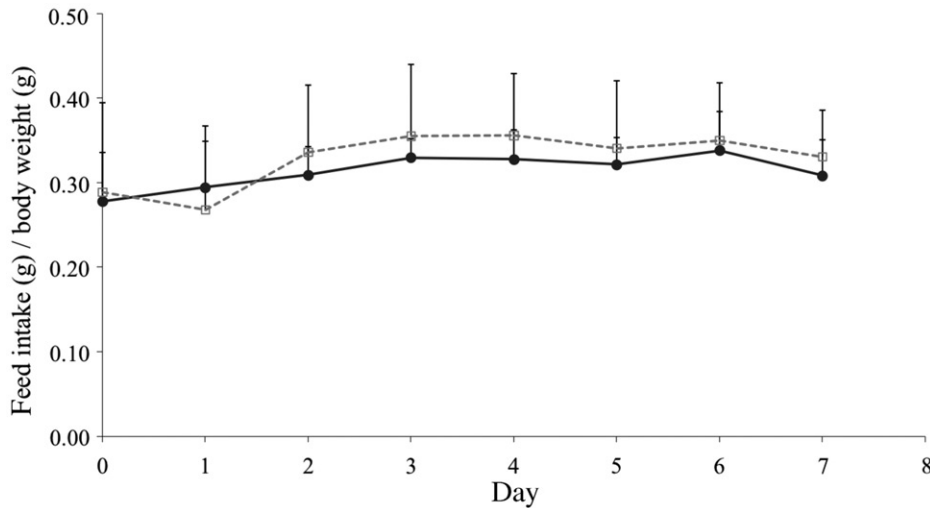
Figure 3. *Chickens' water consumption per gram of BW. In the figures, the solid line represents CG variation in body weight gain and the dashed line represents CHG variation. Error bars represent standard deviations of experimental data from the 6 cages of three animals each. *Statistically different from CG.*
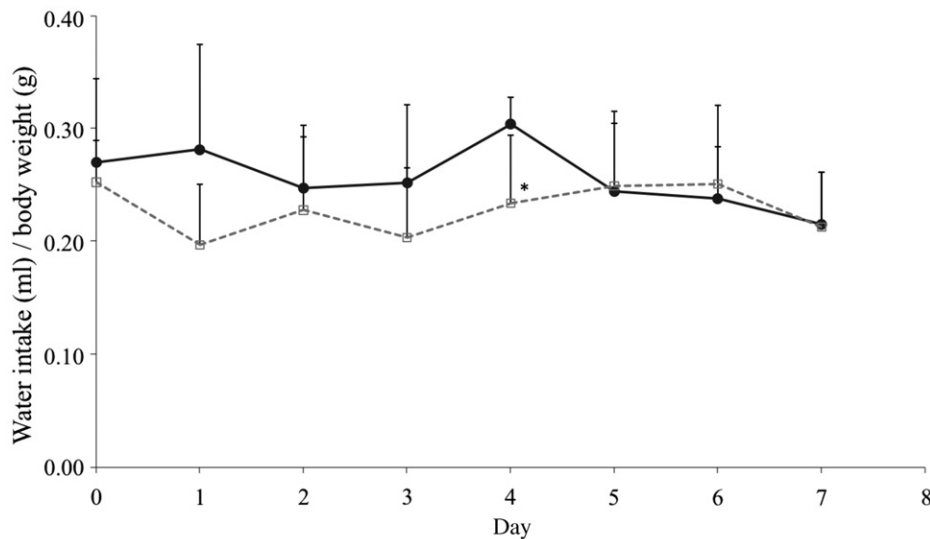
Osburn (1980), the lethal dose of *Escherichia coli* endotoxins to chickens is ≥ 50 mg/kg or $\geq 5 \times 10^8$ EU/kg (1 EU/ml ≈ 10 ng/ml). Kokosharov (2002) reported that little is known about LPS activity in chickens and sometimes experimental data are conflicting and divergent. The phage lysate we administered has 10 times the volume, and therefore might have about 10 times the LPS content of the phage cocktails used for therapeutic purposes by Huff *et al.* (2004, 2005). These authors did not observe any harmful effects on chickens' health and advise phage concentration in therapeutic mixtures ranging between 10^7 and 10^9 pfu/ml (Huff *et al.*, 2004, 2005).

During the *in vivo* trial, the birds challenged with the phage lysate, as compared to the CG, exhibited prostration and decreased feed and water intake only during the day of inoculation: subsequently, chickens' behaviour did not show visual differences between groups. Similar findings were reported by Smith *et al.* (1978) during an experiment with endotoxins from *Salmonella enterica serovar Gallinarum* administered to 14-d-old chicks. Despite the observation of some clinical illness without mortality a few hours after intravenous injection of 1.5×10^7 ng/kg LPS, most of the responses returned to normal within 24–48 h. Also Kokosharov (2002) observed illness in cockerels one hour after injection of 5.0×10^7 ng/kg LPS. Birds were described as standing in the corners of the cages with signs of depression, reluctance to move, somnolence,

loss of thirst and appetite, and diarrhea, which all gradually disappeared. This author did not report any death among the cockerels challenged.

In the present work, one chicken died one hour after the intramuscular inoculation, probably due to anaphylactic shock, as no visible lesions were found at necropsy. The statistically significant decrease of BW gain in CHG at day 1 was probably due to the chickens' prostration and apparent loss of appetite. On day 6, the differences between groups may be explained by the occurrence of an unexpected factor during the experiment: some feeders of the CG were found empty in the morning, for the first time since the beginning of the housing. As feed had to be provided *ad libitum*, feeders were immediately refilled. This happened a few hours before birds weighing and thus might had contributed to the higher average weight in the CG. Regarding water intake, the non-significant decrease on day 1 in CHG was probably due to the birds' prostration. There is no explanation for the significant decrease in water intake on day 4. However, because water intake was thereafter similar in the two treatments, this difference was probably not meaningful.

The absence of macroscopic lesions in the internal organs of the euthanised birds suggested that the phage lysate did not cause any visible internal injurious effect.

For pharmaceutical purposes there are many studies evaluating endotoxic effects in humans and other animals, like toxicity evaluation of antibiotic-induced endotoxin release, water purification for dialysis, and so on (Mathison & Ulevitch, 1979; Shenep et al., 1985; Røkke et al., 1988; Natanson et al., 1990; Friedland et al., 1993; Martich et al., 1993; Nakamura et al., 1998; Brüssow, 2005). However, the variation in sensitivity to endotoxin among species and the higher resistance of chickens to endotoxin effects as compared to mammals (Butler et al., 1977; Smith et al., 1978; Culbertson & Osburn, 1980; Jones et al., 1981), means that results from trials with other animals are of limited relevance to our findings.

In conclusion, apart from the immediate short term reduction in activity, no adverse effects were found in chickens challenged by phage crude lysate containing 8.21×10^4 EU/ml endotoxins, suggesting that phage crude lysate is not toxic for chickens.

ACKNOWLEDGEMENTS

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