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Researcher Contact Details

A/Prof Shubiao Wu
School of Environmental and Rural Science
University of New England
Armidale NSW 2351 Australia
Email: swu3@une.edu.au
Phone: +61 2 6773 2238

Dr Nalini H Chinivasagam
EcoSciences Precinct
Department of Agriculture and Fisheries,
Dutton Park 4102, QLD, Australia
Email: nalini.chinivasagam@daf.qld.gov.au
Phone: +61 7 3255 4301

Professor Robert Moore
School of Science
RMIT University
Bundoora VIC. 3083 Australia
Email: rob.moore@rmit.edu.au
Phone: +61 3 9925 7580

In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

Poultry CRC Ltd Contact Details

PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

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Executive Summary

Necrotic enteritis (NE) is one of the world's most prominent and severe poultry diseases. The European Union has enforced a ban on the use of in-feed antibiotics, and consumer pressure in other regions may force similar restrictions on antibiotic use. Furthermore, the move from caged or housed chicken farming to free range tends to lead to more difficulties with disease control. Therefore, alternative strategies for the control of NE are needed to reduce the health and economic impact of the disease. Bacteriophages are naturally existing viruses of bacteria. They have been investigated for their application as an antibiotics replacement to control infectious diseases and it has been shown that the use of bacteriophages in controlling diseases in animals is promising.

In the current study, 18 Clostridium perfringens phage strains were isolated, purified and enriched. The analysis of whole genome sequences from five strains indicated that the strains were 99%-100% identical. The genomes of sequenced strains were homologous to a Clostridium phage in the database but only showed a similarity of 72% indicating that we have isolated a group of novel Clostridium phages. Seventy-seven genes were predicted in the genome and 19 related to phage functions.

In vivo experiment showed that challenge with *C. perfringens* produced subclinical NE in the birds, evidenced by the mild lesion in the intestine and poorer performance compared to the control. Less severe intestinal lesion in the phage treated birds suggests the gavages of 106 units phage following *C. perfringens* can to some extent protect birds from NE although performance of the birds was not improved statistically. This shed lights on the possible use of phage to protect chickens from NE occurrence under Australian rearing system and Australian environment. Further investigation of the effect of phage treatment to the birds and application protocol is warranted.

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Introduction

Necrotic enteritis (NE) is one of the world's most prominent and severe poultry diseases. The European Union has enforced a ban on the use of in-feed antibiotics, and consumer pressure in other regions may force similar restrictions on antibiotic use. Furthermore, the move from caged or housed chicken farming to free range tends to lead to more difficulties with disease control. Therefore, alternative strategies for the control of NE are needed to reduce the health and economic impact of the disease.

Clostridium perfringens is the causative agent of NE. Much work has been conducted by the Poultry CRC in NE, including the effort towards producing both killed and live vaccines (Moore, pers. Comm.). Whilst vaccines are an obvious tool to address NE, there are a number of other options that may be worth investigation. One such option is the use of bacteriophages. Although there is much interest in finding bacteriophages against all sorts of organisms, little information is present to show the control of NE *in vivo* in chickens or other poultry, particularly in the Australian environment. A few groups have conducted bacteriophage research, and several phage and lysin products are patented. Such products, however, have not been explored using a production relevant NE model like the one in operation at UNE.

Bacteriophages are naturally existing viruses of bacteria, and it is estimated that more than 5000 classes of phage are present on earth (Ackermann, 2011). In humans, bacteriophages have been used as antibacterial agents for more than a century since the report by Hankin (1896). They were used for the treatment of soldiers infected with various bacterial diseases by the Soviet Union military during World War II (Abedon, et al., 2011). Due to the wide use and effectiveness of antibiotics, phage therapy declined after World War II and it was only in Eastern European countries and the Soviet Union that the development of phage therapy was actively supported until the 1990's. In contrast, research into phage therapy really didn't take off in western countries until the 1990's. In livestock animals, bacteriophages have recently been investigated for their application as an antibiotics replacement to control infectious diseases (Garcia, et al., 2007; Kang, 2013; Karthik, et al., 2014), due to the development of antibiotic resistance in bacteria. In poultry, bacteriophages targeting *Salmonella* Gallinarum*, E .coli*, and *Campylobacter jejuni* have been isolated by several groups (Jamalludeen, et al., 2009; Kwon, et al., 2008; Loc Carrillo, et al., 2005). It has been shown that the use of bacteriophages in controlling diseases in animals is promising.

A few attempts to use bacteriophages or their lytic enzymes to control NE in poultry have been reported in the literature (Gervasi, et al., 2014; Miller, et al., 2010; Nariya, et al., 2011; Seal, 2013; Zimmer, et al., 2002). It appeared that the phages are effective in controlling NE with a reduction in mortality of up to 92% compared to untreated NE birds (Miller, et al., 2010). Other efforts involved the use of phage originated hydrolase or endolysin and *in vitro* experiments showed the effectiveness of the enzymes to control *C. perfringens*. Patent search have been performed through Google patent and SciVerse Hub (via Scopus) and 15 patents were found to be related to the control of *C. perfringens* by bacteriophages or their derivative enzymes, for example, Mathers and Sulakvelidze (2010), Pasternack and Sulakvelidze (2009), Son, et al. (2014), and Zimmer, et al. (2008). Among those, the inventions involved the purification of phages or lysins, or sequencing of the genes coding the lysins. Despite the *in vitro* lytic activity of the phages and lysin against *C. perfringens*,

only Mathers and Sulakvelidze (2007) showed phage protection of birds from NE under their challenge conditions. Extensive application of phages or derivative enzymes has been lacking, especially in a wide range of NE challenge models. To be more applicable and acceptable on an industrial scale, bacteriophage treatments of chickens infected with NE disease should be investigated further, especially under different rearing systems and in different countries, in which *C. perfringens* strains may be different. Previous in vitro work to determine the sensitivity of *C. perfringens* strains to various phage isolates have used a diverse and largely uncharacterized set of *C. perfringens* strains (Gervasi, et al., 2013; Morales, et al., 2012). It has been usual to find that particular phages only lyse a subset of the strains tested. With our growing knowledge of the pathogenesis of *C. perfringens* strains we are now in a position to concentrate the search for phage on those that specifically attack pathogenic strains. Activity against non-pathogenic strains is not important and it could even be argued that phage that lyse pathogenic strains but leave non-pathogenic strains intact would be the most desirable products. Therefore, bacteriophage that specifically target strains expressing the NetB toxin are likely to be critical for the control of disease.

Objectives

The aim was to search for new phage isolates that can lyse a range of *netB* positive *C. perfringens* strains. This would demonstrate whether certain phage or combination of phages can offer protection against NE outbreaks. We aimed to isolate bacteriophages specific to *C. perfringens* strains isolated from Australian poultry environments and investigate the possibility of employing such phages to control NE diseases in a broiler challenge model established at the University of New England.

Methodology

Sample collection

Environmental samples were collected in the backyard chicken shed, sewage outlet points of chicken farms and intestinal content of scarified chickens in Armidale and Tamworth of New South Wales, Australia.

Clostridium perfringens isolation

C. perfringens (Cp) strains from the collected environmental samples were isolated followed by culturing the diluted samples on TSC agar. Briefly, Tryptose Sulphite Cycloserine (TSC) selective agar was prepared following manufacturer instruction, autoclaved and kept at 60°C. TSC supplement dissolved in sterilised water and Egg Yolk Emulsion were added and mixed gently, and agar plates were prepared for Cp culture. The samples were streaked onto the agar plates in duplicates plates and the plates were packed in sealed plastic bags with an anaerobic sachet. The plates were then incubated at 39ºC for 48 hr or until separate colonies were visible. A single colony from the TSC selective agar plates was picked and sub-streaked onto a fresh TSC agar for further purification of the bacteria strain. The plates were again incubated at 39°C for 48h or until the colonies were viable and stored at 4°C no more than 14 days.

Isolation and enrichment of bacteriophage from the samples

Collected environmental samples were immediately brought to the lab. Approximately 1g of dry sample or 1ml of the wet sample were aseptically suspended into 1ml of sterile water and mixed gently. A sterilised culture loop was then used to dip the water-sample mixture and streaked onto a perfringens selective agar (Perfringens Agar Base (Oxoid) with the addition of egg yolk emulsion and appropriate supplementation of either Tryptose Sulphite Cycloserine (TSC) or Shahidi-Ferguson Perfringens (SFP)). Grown *C. perfringens* colony underwent another round of subculturing on the same selective agar. The positive perfringens colony was streaked onto fresh Horse Blood Agar (HBA) with duplication from each colony and grown at 39ºC for 18 hrs. Grown colonies were harvested into 3 mL brain heart infusion (BHI) broth then an adequate amount was transferred to a fresh 4ml BHI broth to adjust turbidity to approximately 220 ± 15 NTU. Bacteriophage samples were prepared by gently shaking 1g raw samples or 1ml liquid sample in the 9ml of SM buffer (0.1 M NaCl, 1 mM $MgSO_4$, 0.2 M Tris-HCl, pH 7.5) for 12 hrs. The supernatant was centrifuged at 11,000 × g at 4 ºC for 5 mins. The resulting clear supernatant underwent filtration through a 0.22-µm-pore-size Millipore filters. The filtrate was prepared into serial dilutions up to 10^{-8} dilution of the original filtrate using SM buffer as the diluent. Then, to each phage dilution to be tested, 200 µl of perfringens culture with adjusted turbidity was mixed with 100 µl of bacteriophage dilutions. The mixture of host and phage was incubated at 37 ºCfor 30min for the initial phage-host interaction, and then mixed into the 0.5% BHI soft agar (supplemented with Mg^{2+} and Ca^{2+}) and poured on the BHI base agar. When the soft agar was set, plates were incubated anaerobically at 39ºC up to 48 hrs. Plates were examined, and any plates with clear plagues were identified. The plagues were punched and suspended into 1ml SM buffer. Plagues were stored at 4 ºC overnight to be homogenised. Serial dilutions of the homogenised plague were made and cultured with susceptible perfringens host with

repetition of three times (Seal et al., 2011; Smith, 1959). The bacteriophage plague was subjected to three consecutive rounds of lysate purification. The phage stocks were prepared from three to five confluent plates from the third round of purified phage. Confluent plates were added SM buffer on top of the agar and shaking slowly 10-20 rpm (or equivalent homogenising measure) for 12 hrs. Homogenised supernatants were then filtered through 0.22-µm-pore-size Millipore filters, and resulting filtrates were precipitated at 40,000 × g at 4 ^{o}C for 2h. Pellets from the centrifuging were resuspended in SM buffer to form the phage stock. The phage stock was stored in the dark at 4 ^{o}C.

Lytic activity analysis of phages

All the isolated phages were further propagated using a soft-layer overlay method on BHI agar plates using *C. perfringens* NE18 as the host. The filtered (0.45 µM membrane) phage lysates were titred and all were within the range of 1-7x10^{7} pfu/ml. The phages were used to infect a range of different *C. perfringens* strains to investigate the strain specificity of the phages. The characteristics of the tested *C. perfringens* strains are detailed in Table 1.

Table 1 Characteristics of *C. perfringens* strains

	Source	Year	*netB* gene	Disease	Reference
NE1	Aus	2002	-	No	Sheedy et al. 2004
NE4	Aus	2002	+	Unknown	Sheedy et al. 2004
NE7	Aus	2002	+	Unknown	Sheedy et al. 2004
NE14	Aus	2002	+	Unknown	Sheedy et al. 2004
NE16	Aus	2002	+	Unknown	Sheedy et al. 2004
NE18	Aus	2002	+	Yes	Sheedy et al. 2004
NE21	Aus	2002	+	Unknown	Keyburn et al. 2006
NE31	Aus	2004	+	Yes	Keyburn et al. 2010
NE36	Aus	2010	+	Yes	Keyburn et al. 2013
NE38	Aus	2011	+	Unknown	Lab stock

All strains were isolated from necrotic enteritis diseased birds.

DNA Sequencing

The phage stocks were concentrated by precipitation with polyethylene glycol and DNA was isolated using a phenol/chloroform extraction method. Sequencing libraries of 5 phages were prepared using a Nextera XT kit. The quality of each of the phage DNA sequencing libraries was validated on a Bioanalyzer using a high-sensitivity DNA chip. The libraries were sequenced on an Illumina Miseq instrument using 2x300 paired-end chemistry.

The sequence data generated from the MiSeq were assembled using the A5-miseq assembly pipeline (Coil et al., 2015). The sequences of the phages were aligned using *Clone Manager* Suite V8 (Scientific & Educational Software, NC) using a2 as the reference. Homology analysis of the phage a2 sequence was performed against the *NCBI database* using the blastn program. The genome of phage a2 was submitted to RAST annotation for gene prediction.

Table 2 Composition and nutrients of starter and grower

Ingredients	Starter	Grower
Wheat (%)	37.8	36.6
Sorghum (%)	20.0	26.7
SBM (%)	28.3	20.6
Canola ml (%)	3.50	5.40
Meat & bone meal (%)	3.70	4.10
Canola oil solvent (%)	3.42	3.92
Limestone (%)	0.87	0.72
Dical Phos (%)	1.07	0.77
Salt (%)	0.10	0.10
Na bicarb	0.20	0.20
UNE Vit Pre (%)	0.07	0.07
UNE TM (%)	0.09	0.09
Choline (%)	0.04	0.05
L-lysine HCl (%)	0.40	0.36
DL-methionine (%)	0.29	0.24
L-threonine (%)	0.18	0.15
Nutrient		
ME Poultry kcal/kg	3,000	3,100
Crude Protein %	23.0	20.8
Crude fat %	5.68	6.49
Crude Fiber %	3.23	3.21
Isoleucine %	0.99	0.87
d Arg pou %	1.34	1.16
d Lys pou %	1.27	1.10
d Met pou %	0.59	0.52
d M+C pou %	0.94	0.84
d Trp pou %	0.24	0.20
d Thr pou %	0.83	0.73
d Val pou %	0.94	0.84
NSP insol g/kg	7.62	10.17
Calcium %	1.00	0.90
Phosphorus avail %	0.50	0.45
Sodium %	0.16	0.16
Potassium %	0.95	0.82
Chloride %	0.21	0.21
Magnesium %	0.22	0.21
Selenium mg/kg	0.72	0.72
Zinc mg/kg	242	243
Iron mg/kg	102	104
Copper mg/kg	38.6	38.6
Manganese mg/kg	219	220
Choline mg/kg	1600	1500
Vitamin A IU	16800	16800
Vitamin E IU	113	113
Vitamin K mg/kg	4.2	4.2
Thiamin mg/kg	7.6	7.6
Riboflavin mg/kg	13.1	13.0
Pantothenic acid mg/kg	51.4	58.6
Pyridoxine mg/kg	10.8	10.8
Biotin mg/kg	0.48	0.47
Linoleic 18:2 %	1.73	1.94

In vivo bioassay of phage protective effect on necrotic enteritis

Day-old birds (Ross 308 Broilers) were obtained from Baiada Hatchery at Tamworth, NSW. Birds were housed in 18 pens each contained 11 birds at Kirby Research Station at the University of New England, Armidale. A standard wheat-soybean starter diet formulated to meet the 2014 Ross 308 nutrient specifications was fed to all birds from d 0-10 (Table 2). A grower diet consisting of wheat based formula according to a standardised nutritional specification for the Ross 308 were fed from day 10 to the end of the trial at day 21 (Table 2). Feed and water were available *ad libitum*.

The experiment was designed to perform three treatments, with control group, and necrotic enteritis challenge with or without and phage treatment. On day 9, NE challenge groups were subjected to oral gavage of 1ml vaccine *Eimeria* strains and non-challenge groups were subjected to oral gavage of 1ml PBS treatment. On days 14-15, birds in challenged groups were inoculated with 10^8 CFU of *C. perfringens* NE 18 strain, and then phage treatment birds were gavaged with 10^6 unit of *C. perfringens* specific phage. On day 16, two birds per pen from all the groups were randomly selected, weighed and euthanised by cervical dislocation to perform post mortem analysis and intestinal lesion scoring. On day 21, the experiment was completed and birds were enthanised and disposed of according to the approved protocol by the Animal Ethics Committee of the University of New England. Pen bird weight and feed intake were measured on days 9 and 21. Feed conversion ratio was calculated by pen feed intake divided by pen bird weight and adjusted with mortality.

Results

Isolation and purification of the *Clostridium perfringens* phage

Eighteen *Clostridium perfringens* phage strains were isolated and enriched. Phages lytic to the *C. perfringens* hosts were observed (Figure **1**).

Figure 1 ***Clostridium perfring*ens phage plaques.** Arrows showing the plaques produced by the strains from sample E that was collected from chicken caecal content.

Phage lytic profile to virulent *C. perfringens* strains

The lytic analysis showed that all 18 phages infected and lysed *C. perfringens* strains NE14, NE18, NE21 and NE36 cells, while *C. perfringens* trains NE1, NE4, NE7, NE16, NE31 and NE38 were all resistant to infection by all the phages.

Sequences of 5 phage strains and alignments

The assemblies resulted in the generation of a single contig for all phages with the same GC content (34%) and very similar genome size (52792-52794 bp) (Table 3).

Table 3 A5-miseq assemblies of the phages

Strains	Contigs	Genome Size	Longest Scaffold	N50	bases >= Q40	% GC
Phage a2	1	52794	52794	52794	52787	34
Phage c2	1	52795	52795	52795	52775	34
Phage d1	1	52794	52794	52794	52775	34
Phage e1	1	52792	52792	52792	52750	34
Phage h1	1	52794	52794	52794	52794	34

Their sequence identities were 99-100% (Table 4). The five phage strains were essentially identical.

Table 4 Alignment statistics of phage assemblies

Starin	Start	End	# Match	NonMatch	Match (%)
Phage a2	**1**	**52794**			
Phage c2	**1**	**52795**	**52794**	**1**	**99.99**
Phage d1	**1**	**52794**	**52792**	**2**	**99.99**
Phage e1	**1**	**52792**	**52789**	**7**	**99.98**
Phage h1	**1**	**52794**	**52794**	**0**	**100**

Homology of the phage genome and gene prediction

Homology analysis of the phage a2 sequence was aligned to *Clostridium* phage vB_CpeS-CP51 with a homology of 72% (Figure 2). Seventy-seven genes were predicted, of which 19 had phage related functions.

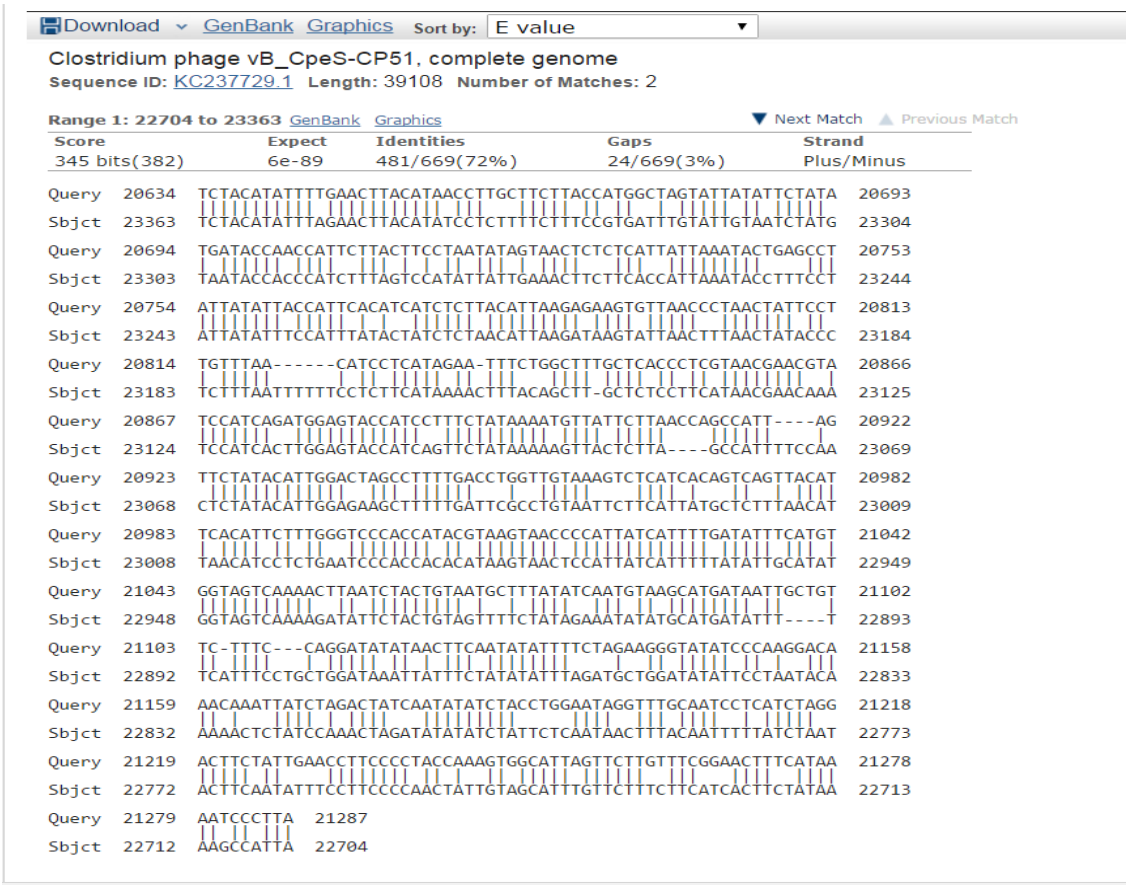

Figure 2 Blast analysis of phage a2 against the NCBI database using the blastn program.

Performance of the birds

Performance of the birds prior and post NE challenge are shown in Table 5. On the day of arrival, birds were evenly distributed in the respective pens designated for different treatments and no difference of body weight (BW) between the groups designated for respective treatments. Prior to the inoculation of *Eimeria* during days 0 to 9, no differences of body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were detected among the groups designated for treatments.

Following *Eimeria* inoculation at day 9 and *C. perfringens* challenge at days 14 and 15, BWG, FI and FCR were significantly affected by the challenge. The challenged birds with or without phage treatment had reduced BWG and FI, and poorer FCR during days 9 and 21 or 0 and 21. This indicated the successful challenge of birds and the challenge produced subclinical NE in the birds. However, phage treatment did not show significant protection of the birds from worsened performance. Only numerical improvement was observed in BWG and FI.

Table 5 Performance of the birds in response to the NE and phage treatments*

Treatment	Control		NE only		NE + phage		ANOVA
	Mean	SE	Mean	SE	Mean	SE	P
Day 0							
BW	37.2	0.3	37.0	0.3	36.8	0.2	0.602
Day 0- 9							
BWG	239	3	244	2	243	1	0.308
FI	206	2	210	3	208	2	0.471
FCR	1.021	0.029	1.015	0.031	1.011	0.013	0.797
Day 9-21							
BWG	748[a]	18	500[b]	12	516[b]	20	0.000
FI	927[a]	22	757[b]	14	775[b]	31	0.000
FCR	1.238[b]	0.010	1.516[a]	0.082	1.511[a]	0.180	0.001
Day 0-21							
BWG	950[a]	21	707[b]	13	722[b]	20	0.000
FI	1128[a]	22	957[b]	14	974[b]	30	0.000
FCR	1.188[b]	0.013	1.355[a]	0.056	1.353[a]	0.118	0.002

* Means within the same row with same letters are not significantly different;
SE: standard error.

Intestinal lesion score and mortality

Mild lesions in the intestinal sections were observed. Lesion scores are summarised in Table 6. Higher lesion scores were recorded in both duodenum ($P < 0.01$) and jejunum ($P < 0.01$) of the birds challenged with NE than those unchallenged. Phage treatment showed protective effect on the infection of NE in birds as phage treatment of the birds had lower lesion scores than those without treatment ($P < 0.01$) and no different lesion scores were observed between control and phage-treated birds. No mortality of birds was observed thus a subclinical NE challenge was achieved.

Table 6 Lesion scores of the birds under treatments at day 16.

Treatment	Duodenum		Jejunum		Ileum	
	Mean	SE	Mean	SE	Mean	SE
Control	0.0[b]	0.0	0.0[b]	0.0	0.0	0.0
NE only	0.8[a]	0.1	0.7[a]	0.1	0.6	0.3
NE + phage	0.2[b]	0.2	0.1[b]	0.1	0.3	0.2
ANOVA (P)	0.005		0.005		0.095	

* Means within the same row with same letters are not significantly different; SE: standard error.

Discussion of Results

The current study isolated and purified *C. perfringens* phages, the genomes of the phages were sequenced and the blast in the NCBI database indicated the phages isolated aligned with the *Clostridium* phage vB_CpeS-CP51 with a homology of 72%. The phages were lytic to four virulent *C. perfringens* including two used in the NE challenge model developed at the University of New England. An in vivo challenge trial was performed to test the protective effect of the phages obtained. The results showed that the phage treatment prevented the challenged birds from developing lesion to the level of the birds without treatment. However, the performance of the birds were not improved by the phage treatment. In the current NE challenge model, *Eimeria* was used to predispose chickens to succumb NE. Evidence showed that *Eimeria* infection of birds may be related to damage of the epithelium releasing serum and other nutrients (Van Immerseel et al., 2009). While *C. perfringens* phage controls the replication of the bacterium in gut and thus leads to lesser severity in intestinal NE lesion, the epithelial damage applied by *Eimeria* may have not been alleviated to such an extent that performance of birds being improved to the level equivalent to the unchallenged control. Indeed, in the current study, no statistical improvement of performance was observed compared to the challenged birds without treatment. However, numerical improved BWG, FI and FCR during 14-21 d of experiment were observed. The short period of trial was due to the consideration that NE occurrence in the birds is usually during 3-4 days following challenge. Therefore, the protective effect of phage to birds would be observed shortly after the challenge and thus improved performance would have been detected at 21 d. This, however, may not be the case when *Eimeria* effect on performance of birds overwhelms. Nevertheless, whether longer experimental period, for example, up to 35 d, may lead to significant improvement of performance warrant further investigation.

The successful isolation and enrichment of the phages lytic to *C. perfringens* strains demonstrated that the protocol development in the current study is effective in isolation of phages in animal and environmental sources. The protocol can then be used in the future studies in isolation of *C. perfringens* phages. However, more diversified sampling sources are needed for isolation of different phage strains that are lytic to different *C. perfringens* strains. This will be more relevant in a wider range of application to protect birds from infection of NE in different outbreak circumstances.

Visible intestinal lesion and worsened performance in the NE challenged birds indicate that the challenge of birds under our experimental conditions was successful and a subclinical infection was achieved. Although phage treatment of the challenged birds did not improve the performance of the birds, less severity of intestinal lesion in the treated birds suggests the phage gavages following *C. perfringens* can to some extent protect birds from NE under Australian rearing system and caused by the field *C. perfringens* strains isolated from Australian farms. The dosage and way of phage administration to birds are also critical factors for the effectiveness of the phage treatment to protect birds from NE infection. However, these are not thoroughly investigated to the best of our knowledge. Therefore, further investigation of the effect of phage treatment to the birds is warranted.

Implications

The current study isolated 18 *Clostridium perfringens* phage strains. The genome sequences of isolated strains indicated that the phages are close to a *Clostridium* phage but only show 72% similarity thus indicating the phages are novel. The phages were used to treat birds challenged by a virulent *C. perfringens* strain. The in vivo experiment showed protective effect of the phage on birds by alleviating lesion in the gut. However, performance was not improved indicating the need of further investigations on the diversity, dosage, and administration protocol.

Recommendations

- Phage isolation protocol can be used in later studies.
- Phage treatment of birds to alleviate NE is a potential method in post antibiotic era.
- Further study is needed to isolate more phages lytic a variety of *C. perfringens strains*. This should involve the collection of samples in more chicken farms, backyards and sewage outlet where poultry farms locate so as to isolate more *C. perfringens* phages. The rest of work will be similar to what have been performed in the current study but the scale will need to be increased so that more diverse phage strains can be isolated for making cocktails lytic to a wide spectrum of *C. perfringens* strains in particular those isolated from NE infected birds in the field. A three year project will more appropriate.
- Further study is also needed to fine-tune the dosage and administration protocol. Once aforementioned cocktails have been formulated, 10^6, 10^7, and 10^8 units of *C. perfringens* phage can be administrated to the birds in challenge trials by gavage, and an estimated level of ~10^6 – 10^8 units of phage per day per bird delivered to chickens through drinking system from the day of challenge for at least 5 days.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Bacteriophages to control necrotic enteritis in broiler chickens
Poultry CRC Sub-Project No.:	1.3.4
Researcher:	A/Prof Shubiao Wu, Dr Nalini Chinivasagam, and Prof Robert Moore
Organisation:	University of New England, Queensland Department of Agriculture and Fisheries, and RMIT
Phone:	+61 2 6773 2238
Fax:	+61 2 6773 3922
Email:	swu3@une.edu.au
Sub-Project Overview	The sub- project was to search for antibiotic alternative agent called phage, a kind of virus that can damage bacteria, in this case, *Clostridium perfringens*. *C. perfringens* is a bacterium that causes a gut disease called necrotic enteritis. Due to the removal of antibiotics in chicken feed, this disease can be devastating. Our study demonstrated that we isolated several novel and nearly identical *C. perfringens* phage strains. The phages obtained can to some extent protect birds from NE infection： less gut lesion in the treated birds. However, no benefits on growth and feed efficiency were detected. We conclude that it is potential to isolate and use phages specifically damaging C. perfringens so that the control NE under Australian rearing broiler system will be possible by using phage.
Background	Necrotic enteritis (NE) is one of the world's most prominent and severe poultry diseases occurring in gut. The European Union has enforced a ban on the use of antibiotics in animal feed, and consumer pressure in other regions may force similar restrictions on antibiotic use. The move from caged or housed chicken farming to free range tends to lead to more difficulties with disease control. Therefore, alternative strategies for the control of NE are needed to reduce the health and economic impact of the disease. Bacteriophages, a kind of virus that can damage bacteria, are naturally existing. They have been investigated for their application as an antibiotics replacement to control infectious diseases and it has been shown that the use of bacteriophages in controlling diseases in animals is promising.
Research	In the current study, 18 *Clostridium perfringens* strains were isolated. The analysis of whole genome (DNA) sequences from five strains indicated that the strains were 99%-100% identical. They were similar to a *Clostridium* phage in the database, but only have a similarity of 72%. This indicates that we have isolated a group of new *Clostridium* phages. Also,

	77 genes were predicted in the genomic (DNA) sequence and 19 related to phage functions. Animal trial showed that infection of the birds with *C. perfringens* produced mild (or subclinical) NE. This can be seen by observed mild lesion in the gut and poorer growth and feed conversion. Less severe gut lesion in the phage treated birds shows that the phage treatment can to some extent protect birds from NE infection, but performance of the birds was not improved as expected. This indicates that it is possible to use phage to prevent chickens from NE infection under Australian rearing system and Australian environment. Further study can be done to investigate the effect of phage treatment to the birds and to develop the application protocol for the broilers.
Sub-Project Progress	Progressed well and completed.
Implications	This study isolated new *Clostridium perfringens* phage strains. These phages were used to treat birds challenged by a *C. perfringens* strain that can cause NE in birds. The animal trial showed protective effect of the phage on birds by reduce severity of lesion in the gut. However, performance was not improved indicating the need of further investigations on the diversity, dosage, and administration protocol.
Publications	No