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Executive Summary

The project aimed to screen various field materials, i.e. faeces or sewage from poultry farms for presence of bacteriophages for *E.coli*, *Pasteurella multocida* (*P.m.*) and *Clostridium perfringens* (*C.p.*).

The bacterial host isolates included a total of six *E.coli* isolates (provided earlier to UNE by Dr. Rob Moore, CSIRO), nine *P.m.* isolates (kindly provided by Dr. Pat Blackall, QPIF, DEEDI and Phil Ashby, Birling Avian Laboratories) and three pathogenic *C.p.* isolates, kindly provided by Dr. Rob Moore, CSIRO.
The methods used for isolation of phages from field materials were adapted from Adams (1959) and Goh et al. (2005) in such a way that faecal or sewage material was directly added to host broth which was grown to the log-phase overnight and then incubated for another 16-18 hours before testing the supernatant for presence of phages on specific agar plates.

From a total of ten field samples which were subject to screening for presence of *E.coli* phages, 2 phages were successfully isolated, bulked up and stored for future use.

A total of nine field samples were screened for *P.m.* phages and there was one potential phage candidate present in five of the nine *P.m.* isolates. However, this potential phage could subsequently not be subcultured or recaptured from the plates. Five field samples were obtained to be tested for presence of *C.p.* phages, however, no phages were detected or isolated.

Contents

Introduction

The purpose of this project was to screen faecal and/ or sewage samples from poultry farms for the presence of phages against *E.coli*, *Pasteurella multocida (P.m.)* and *Clostridium perfringens (C.p.)*, isolate potential candidates, grow them to bulk amounts of high titre and store materials for future use.

The rationale for this project is based on the following facts and previous research in other species than poultry.

Given the increased incidence of antibiotic resistance and increased pressure to ban their use for non-therapeutic purposes in livestock, alternative approaches to controlling conditions such as necrotic enteritis are increasingly being sought. Much work has been carried out on probiotics and prebiotics for this purpose but another potential tool in the non-antibiotic armoury would be the therapeutic use of bacteriophages. Bacteriophages are viruses which infect bacteria, and are highly specific to one bacterial host. They are nontoxic to animals and plants; and they usually increase in titre as they infect, multiply in, and kill their target microbes (Summers, 2001). Phage therapy has previously been used effectively in the former Soviet Union and Eastern Europe to treat Shigella and Staphylococcus infections in humans (Babalova et al. 1968, Kucharewicz- Krukowska e al., 1987, Slopek et al., 1987). In theory at least phages could be supplied to chickens via feed or water in a prophylactic or therapeutic fashion. Application may therefore extend to therapy for treatment of infections such as with *C.p.*, pathogenic *E. coli* or *P.m.*

A recent study on the use of *E. coli* bacteriophages in poultry indicated that they can provide an alternative treatment to the use of antibiotics (Huff et al., 2006). However, to date, no bacteriophages have been detected or described which could be used to treat *C.p.* infections in chickens. In the light of the above, phage therapy shows considerable potential for treatment of not only *C.p.* infections but also other bacterial infections of chickens, e.g. colibacillosis or pasteurellosis. Development of effective phage therapy for any of these conditions would be an enormous asset to the poultry industry.

Objectives

The objectives of this project were:

1. To isolate bacteriophages against *E.coli*, *Pasteurella multocida* and *Clostridium perfringens* from poultry field materials, mainly faecal samples and sewage.
2. To determine their efficacy *in vitro* by titration
3. To bulk up phage material and preserve for future use.

Methodology

Bacterial isolates

E.coli

Six E.coli isolates had been kindly provided by Rob Moore, CSIRO previously and had been stored in glycerol stocks at UNE. The six isolates E3, E30, E133, E956, E1043 and E1292 were originally isolated from field samples and ranged from highly virulent to avirulent. All isolates were tested for their pathogenicity in previous experiments (Ginns et al., 1998; Tivendale et al., 2004).

Pasteurella multocida

The following 6 *P.m.* isolates were kindly obtained from Dr. Pat Blackall, QPIF, DEEDI:
PM 1239 – serovar 1 chicken liver NSW
PM 1244 – serovar ¼ cross reacting chicken liver VIC
PM 1260 – serovar 1 chicken ovary SA
PM 1267 – non-typable chicken lung QLD
PM 1315 – serovar 1 chicken peritoneum VIC
A further 3 *P.m.* isolates were obtained from Phil Ashby, Birling Avian Laboratories. These 3 isolates were isolated from birds of different flocks with confirmed *P.m.* infection, but had not been typed or tested any further.

Clostridium perfringens

The three *C.p.* strains Longford1, Musdale1 and PA1 were obtained from Rob Moore, CSIRO. All isolates were isolated from cloacal swabs from broiler flocks.
Longford1 - Tasmania, October 2008.
Musdale1 - Queensland, September 2008.
PA1 - Queensland, November 2008.

Media used for bacterial isolates

E.coli

- McKonkey Agar, (Oxoid) was used to subculture pure *E.coli* isolates.
- Brain heart infusion broth (Oxoid) was used to produce overnight cultures of *E.coli*
- Brain heart infusion agar (Oxoid) was used in attempt to isolate phages.

Pasteurella multocida

- Brain heart infusion agar (Oxoid) with 5% defibrinated horse blood was used to subculture pure *P.m.* isolates and to isolate phages.
- Brain heart infusion broth (Oxoid) with 5% defibrinated horse blood was used to produce overnight cultures of *P.m.*

Clostridium perfringens

- TSP agar (Oxoid), was used to subculture pure *C.p.* isolates and to isolate phages.
- Reinforced Clostridial medium (Oxiod) was used to produce overnight cultures of *C.p.*

All media and broths were prepared according to manufacturer's recommendations, aliquoted as needed into 10 ml McCartney or Schott bottles and autoclaved prior to use.

Phage storage media

For 100 ml storage media use:

NaCl	0.58g
MgSO4*7H2O	0.20g
1M Tris (ph=7.5)	5ml
5% gelatine	200ul

Make this up to 100ml with distilled water and autoclave.

Sample collection

Sewage and faecal samples were collected from commercial flocks with obvious infections of *P.m.* or *E.coli* into sterile 100 ml jars. The samples were kept cool immediately after sampling and sent via courier to UNE Armidale. The samples were processed within 2-24 hours after arrival at Armidale and were kept refrigerated at 4-7ºC throughout.

Phage isolation

The processing of the samples was generally following the methods by Adams (1959) and Goh et al. (2005). However, these methods were modified as follows:

- Fresh sterile overnight broths of all host bacteria were prepared one day prior to the expected arrival of field samples
- 3-5 g or 3-5 ml of original sample was directly added to overnight broths of host bacteria (log phase of growth) and incubated again overnight at 37ºC. The *C.p.* samples were incubated in specific anaerobic jars at the same temperature.
- Another lot of fresh overnight broths of all host bacteria were incubated overnight to log phase.
- Sterile agar stored in 10 ml McCartney bottles were put in a 100◦C oven until the agar was just melted and then transferred into a water bath set at 47ºC to keep the agar liquid.
- The faeces/sewage – bacteria broths were transferred into sterile 10 ml centrifuge tubes and centrifuged in a tabletop centrifuge at 3000 rpm for 25 min.
- The supernatant was filtered through a sterile 0.45 µm syringe filter into another sterile 10 ml centrifuge tube. This supernatant was used to inoculate the specific agar plates. It was kept refrigerated at 4ºC until used.
- To each McCartney bottle containing the specific agar, 100 ul of fresh host bacteria and 1ml of filtered supernatant was added, mixed thoroughly and poured onto a sterile Petri dish, allowed to settle and was then incubated overnight at 37ºC.
- The next morning, plates were inspected for presence of phages by comparing the plates against a positive control containing only host bacterium.
- In case of inhibition of host bacteria, the plate was covered with 4 ml of phage storage medium and allowed to incubate at room temperature for 3-4 hours.
- The supernatant from the plate was recaptured and transferred into a sterile centrifuge tube and centrifuged in a tabletop centrifuge at 3000 rpm for 25 min.
- Using a sterile Pasteur pipette, the supernatant was transferred into a new sterile centrifuge tube and again 1ml supernatant poured onto agar plates together with 100 ul fresh host bacteria in order to bulk up the phages.
- 10-fold serial dilutions were then made in order to titrate the phage material.
- Any isolated phages were stored in phage medium at 4ºC.

Determination of phage titre

In order to determine the titre of phages, 10-fold serial dilutions were made. Plaques were counted on the plate which allowed for easy plaque enumeration and multiplied with the reciprocal value of the the dilution at which plaque counts were performed. Phage titre is given in plaque forming units, PFU/ml.

Results

E.coli

From a total of 10 field samples submitted to UNE, 2 phages were successfully isolated and bulked up for storage. The 2 phages could repeatedly be recovered and subcultured, even after several weeks of storage at 4ºC in phage storage medium. Plate 1 shows a typical agar plate with almost complete inhibition of bacterial growth compared to a positive control plate with only host bacteria.

Both phages were titrated on agar plates and titres ranged from 10^3-$10^{5.6}$ PFU/ml. Phage material was bulked up and stored in phage storage media at 4◦C for future use.
An additional potential 3rd phage was isolated in late December 2009, but wasn't bulked up and titrated. Material of this phage candidate was stored at 4ºC for future work.

Plate1: Plate showing growth inhibition of *E.coli* when co-infected with phage solution isolated from field material (left) compared to a plate containing only host bacterium (right).

Pasteurella multocida

From a total of nine field samples submitted to UNE, one potential phage candidate inhibited growth in five of the nine host isolates used. However, this potential phage could not be subcultured and bulked up to high titre.
Plate 2 shows the growth inhibition in *P.m.* compared to a positive control containing only host bacteria.

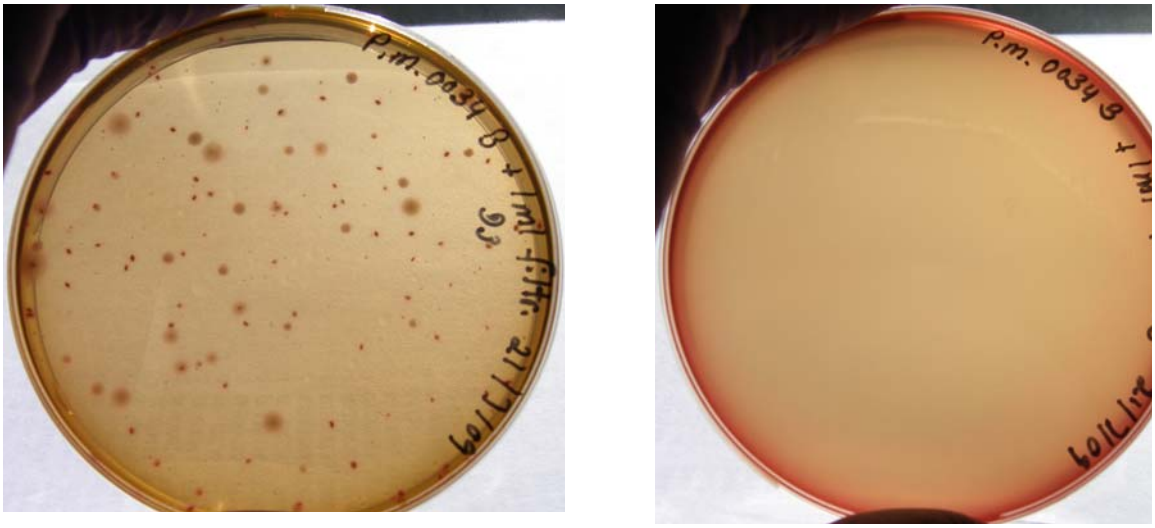

Plate 2: Plate showing growth inhibition of *P.m.* when co-infected with phage solution isolated from field material (left) compared to a plate containing only host bacterium (right).

Clostridium perfringens

From a total of 5 field samples to be tested for presence of phages against *C.p.*, none showed any promise for presence of phages. All three *C.p.* isolates grew to a complete layer on the agar plates and no growth inhibition could be observed with any of the field samples.

Discussion

The aim of this short term project was to isolate bacteriophages for *E.coli*, *Pasteurella multocida* (*P.m.*) and *Clostridium perfringens* (*C.p.*) from various field materials, mainly faeces or sewage from poultry farms. Bacteriophages have several characteristics that make them potentially attractive therapeutic agents against bacterial infections. One of them is the high specificity and effectiveness in lysing targeted pathogenic bacteria thus overcoming the increasing issue of antibiotic resistance. The high host specificity was evident with the 2 isolated *E.coli* phages in this study which were only effective against one *E.coli* isolate at a time. A previous study successfully isolated *E.coli* phages with a broader lytic spectra of *E.coli* strains (Oliveira et al., 2009). These authors isolated five bacteriophages from poultry sewage and tested them against 148 serotyped *E.coli* strains with high patterns of antibiotic resistance (Oliveira et al., 2009) and found that a cocktail of three of those phages showed to be effective against 71% of the *E.coli* strains under test. This suggests that further research should be done in Australia in attempt to isolate bacteriophages with a broader lytic spectrum against pathogenic *E.coli* in poultry. Consequently, the number of bacterial host isolates should be increased in order to widen the isolation success rate from field materials. The same applies to any future work on bacteriophages for *P.m.* and *C.p.* Previous studies both reporting isolation of *P.m.* phages worked with up to 60 bacterial host isolates (Fierlinger et al., 1981; Campoy et al., 2006).

There is a lack of data with regards to the isolation of bacteriophages of *C.p.* is slim, however, work has been done to isolate bacteriophages of *Clostridium difficile* (Goh et al., 2005). Future work should therefore concentrate on investigations as to whether there are no existing *C.p.* bacteriophages or whether previous failure to isolate *C.p.* bacteriophages was a matter of mismatch between the host and highly specific phages the sample material or was due to methodological problems.

Another reason which might have resulted in lower than expected isolation success of phages was the fact that most work was performed during the winter months. Bacteriophage activity is generally only evident when the environmental and nutritional conditions are conducive to growth of the host. At low temperatures, growth rates of pathogens may be much lower and the success of the bacteriophage infection cycle and replication activity, resulting in the death of the host cell, will be less (Adams, 1955). It would therefore be worth to extend the work over a whole summer period in attempt to maximise phage isolation rates.

With regards to the future potential of bacteriophages to prevent bacterial infections in poultry and poultry products, several earlier studies indicated that if the appropriate phages were present at the right time, and at the right titre, they may prevent infection by the organism targeted by the bacteriophage (Barrow et al., 1998; Huff et al., 2002; Higgins et al., 2005). Although there are many issues to be solved with regards to the practical use of bacteriophages, these viruses show tremendous potential to help get some of the bacterial poultry pathogens under control, especially in the face of increasing antibiotic resistance of many bacterial pathogens.

Implications

This project delivered improved methodologies for the isolation of bacteriophages from field materials. It shows promise to help improve the capacity of the Australian poultry industry to manage some of its important endemic diseases given the fact that many poultry pathogens, particularly *E.coli* , are becoming more and more resistant to antibiotics. The work of this project could serve as a template for future isolation of bacteriophages from faeces and sewage materials.

The major implications of this project are:

1. Bacteriophages for poultry pathogens can be isolated from various field materials, bulked up to high titres and stored for future use.
2. The success rate of phage isolation should significantly increase if more host isolates were used for primary isolation.
3. Environmental temperature has a potential effect on natural phage activity and might therefore influence the success rate of phage isolation.

Recommendations

The major recommendations arising from this work are as follows:

1. Further phage isolation work should be performed including a summer period with higher environmental temperatures in order to increase success rate of phage isolation.
2. The number of host isolates used to screen field materials for phage presence should be increased in order to maximise the phage isolation rates.
3. Isolated phages should be tested *in vivo* to determine their value as a therapeutical tool against poultry diseases.

Acknowledgments

I would like to thank Dr. Ben Wells (Cordina), Dr. Phil Ashby (Birling Avian Laboratories) and Sue Sharpe (Birling Avian Laboratories) for the supply of field samples. Also I would like to thank Dr. Rob Moore (CSIRO, Geelong) and Dr. Pat Blackall (QPIF, DEEDI) for supplying the bacterial host isolates.

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Plain English Compendium Summary

Project Title:	Screening for bacteriophages of selected poultry pathogens
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Objectives	The objectives of this project were: **1.** To isolate bacteriophages against *E.coli*, *Pasteurella multocida (P.m.)* and *Clostridium perfringens (C.p.)* from poultry field materials, mainly faecal samples and sewage. **2.** To determine their efficacy *in vitro* by titration **3.** To bulk up phage material and preserve for future use.
Background	Bacteriophages are viruses which infect bacteria, and are highly specific to one bacterial host. They are nontoxic to animals and plants; and they usually increase in titre as they infect, multiply in, and kill their target microbes. Phage therapy has previously been used effectively in the former Soviet Union and Eastern Europe to treat Shigella and Staphylococcus infections in humans. In theory phages could be supplied to chickens via feed or water in a prophylactic or therapeutic fashion. Application may therefore extend to therapy for treatment of infections such as with *C.p.*, pathogenic *E. coli* and *P.m*. A recent study on the use of *E. coli* bacteriophages in poultry indicated that they can provide an alternative treatment to the use of antibiotics. However, to date, no bacteriophages have been detected or described which could be used to treat *C.p.* or *P.m.* infections in chickens. In the light of the above, phage therapy shows considerable potential for treatment of not only *C.p.* infections but also other bacterial infections of chickens, e.g. colibacillosis, or pasteurellosis. Development of effective phage therapy for any of these conditions would be an enormous asset to the poultry industry. This project will screen for the presence of bacteriophages directed against *C.p., P.m.* and *E. coli*.
Research	Methods to isolate phages from field materials were adapted and optimized so that field samples were directly incubated with an overnight culture of the specific host bacterium and then incubated for another 16-18 hours before testing the supernatant for presence of phages on specific agar plates. Potential phages were recovered from the agar plate and bulked up by overnight incubation in an overnight culture of the specific host bacterium, titrated and stored at 4◦C in phage storage medium for future use. The bacterial host isolates included a total of six *E.coli* isolates, nine *P.m.* isolates and three pathogenic *C.p.* isolates. A total of ten field samples were subject to screening for presence of *E.coli* phages, a total of nine field samples were screened for *P.m.* phages and five field samples were obtained to be tested for presence of *C.p.* phages.
Outcomes	From a total of ten field samples which were subject to screening for

	presence of *E.coli* phages, 2 phages were successfully isolated, bulked up and stored for future use. A total of nine field samples were screened for *P.m.* phages and there was one potential phage candidate present in five of the nine *P.m.* isolates. However, this potential phage could subsequently not be subcultured or recaptured from the plates. Five field samples were obtained to be tested for presence of *C.p.* phages, however, no phages were detected or isolated.
Implications	This project delivered improved methodologies for the isolation of bacteriophages from field materials. It shows promise to help improve the capacity of the Australian poultry industry to manage some of its important endemic diseases given the fact that many poultry pathogens, particularly *E.coli* , are becoming more and more resistant to antibiotics. The work of this project could serve as a template for future isolation of bacteriophages from faeces and sewage materials. The major implications of this project are: 1. Bacteriophages for poultry pathogens can be isolated from various field materials, bulked up to high titres and stored for future use. 2. The success rate of phage isolation should significantly increase if more host isolates were used for primary isolation. 3. Environmental temperature has a potential effect on natural phage activity and might therefore influence the success rate of phage isolation.
Publications	Dr Katrin Renz is expected to write a short journal or conference paper on the work arising from this project.