BACTERIOPHAGE TO CONTROL ENTEROTOXIGENIC *E. COLI*

Report prepared for the Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

This study was designed to evaluate the efficacy of a bacteriophage (phage) cocktail for the treatment of diarrhoea caused by enterotoxigenic *Escherichia coli* (ETEC). Neonatal (ND) and post-weaning diarrhoea (PWD) due to ETEC infections are a serious economic and animal welfare issue in pig production. Historically antibiotics have been the standard treatment however there has been world-wide increase in the incidence of infections due to antibiotic resistant strains of ETEC. ND and PWD are complex multifactorial conditions with factors such as genetic susceptibility, weaning age, stress, diet and viral infections implicated in the infection. Alternative prevention and treatment strategies are being investigated and implemented. These include breeding for resistance, vaccination, dietary strategies, increasing weaning age, pre/probiotics and bacteriophage therapy.

Bacteriophages are viruses specific for bacteria only and are harmless to other cells both plant and animal. They are often highly specific to species and even strain level as has been noted in this study. There are two types of life cycle; lytic (virulent) or lysogenic (temperate). It is critical that only lytic phages are used for therapy as they induce rapid death of the host cell. Lysogenic phages can remain within the host in the form of a prophage where the DNA is incorporated into the host genome or as a plasmid. Phage cocktails of more than one phage isolate are recommended reducing the development of resistance.

Phages specific for ETEC strains isolated from scouring piglets at the Rivalea Corowa site were isolated, purified and checked for host range. The first group isolated was specific for toxigenic O157 strains. A pilot study was carried out at the Large Animal Research and Imaging Facility (LARIF) of the IMVS at Gilles Plains. Assessing the efficacy of the phage cocktail was unsuccessful due to the inability to induce PWD with ETEC challenge. Lack of susceptibility to these pathogens was most likely due to the absence of F4 receptors in the small intestine but other factors such as diet, health status and housing could also have been involved. The efficacy of the phage cocktail needed to be assessed on piglets where the infection occurred naturally, that is, at Corowa.

Since development of this cocktail a number of novel ETEC strains appear to have emerged. This dynamic change in pathogen profile has been rapid and has not been reported in previous studies. The forces driving such change have not been elucidated. These new strains were not lysed by phages in the UniSA collection. Changes in management and alternative control procedures were also implemented that have significantly reduced the incidence and severity of ETEC infections in module 3 where the trial was carried out. These include a reduction in agalactia of sows, implementation of different cleaning disinfection agent/protocols and a low protein weaner diet.

Enrichments using effluent and soiled bedding from Corowa modules were carried out and phages specific for 11 of the 19 ETEC isolates were isolated, purified and characterised. A phage cocktail was prepared and administered to three treatment litters and compared to three control litters at onset of scouring. The concern with this approach was that the actual strain/s of ETEC associated with the diarrhoea was not known at the start of the trial. The piglets could be infected with a combination of different ETEC strains some or all of which might not be lysed by the phages.

The results of the trial indicate that more than one ETEC strain was present in the module and only two piglets in one litter carried haemolytic *E. coli* that were lysed by phages in the cocktail. This was the only litter that was likely to respond to the phage cocktail. There was no control group with the same pathogen profile. The efficacy of phage treatment could not be definitively demonstrated without comparison to controls but the phage therapy most likely influenced the reduction in scouring and survival of the remaining piglets in this litter. The data suggests that phages have potential for treatment of ETEC induced diarrhoea but further work needs to be done to confirm the result. The remaining two treatment groups and all three control groups had only non-haemolytic strain/s. The actual number of ETEC strains present was not determined and would require PCR analysis for specific virulence factor genes.

The complication with performing phage therapy trials at Corowa is the number of uncontrollable variables, that is, multiple potential ETEC strains. These phages are very specific and in contrast to antibiotics may have no impact on some ETEC strains present. The recommendation is to carry out a trial at a facility with infections due to one or few known & characterised ETEC strains. Further to this, isolation and characterisation of phages specific for the emerging ETEC strains should be an ongoing process leading to the development of a library of therapeutic phage candidates. ETEC isolates would be screened against phages in the library to formulate an effective phage cocktail for treatment. The experience in module 3 at Corowa emphasises the multifactorial nature of ETEC infections and that good management and husbandry practices can have a significant impact on the outcomes of infection. Bacteriophages have potential for prevention and treatment of ND and PWD in combination with these approaches.

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1. Introduction

Enterotoxigenic *E. coli* (ETEC) is an important infective agent of neonatal and weaned piglets causing diarrhoea leading to serious morbidity, mortality and economic loss. ND and PWD have traditionally been treated using antibiotics however there has been a world-wide increase in the incidence of PWD most notably due to antibiotic resistant ETEC strains. Strategies for prevention and treatment include vaccination, nutritional intervention, feed additives, prebiotics, probiotics, breeding for resistance and bacteriophages.

Bacteriophages are viruses that infect only bacteria and can be highly specific to species and even strain level. They are non-toxic to other organisms, plant or animal, and can survive long term without a suitable host. They increase in number rapidly as they infect and multiply in the host cell, ultimately killing it [1]. They are the most abundant biological entities on Earth with numbers estimated at 10^{31} and are an integral part of any ecosystem coexisting and co-evolving with bacteria [2]. Large numbers of phage and bacteria are found in all but the most extreme environments. Phages and their bacterial hosts are in a dynamic relationship involving cycles of resistance and counter-resistance mutations which inevitably lead to major fluctuations in phage and host populations [1]. Phages are effective against antibiotic resistant bacterial strains.

Therapeutic application of phages has a long history in veterinary and human medicine since their discovery in the early 1900s but with varying degrees of success. The biology and life cycle of phages were poorly understood and crude phage preparations were often contaminated with bacterial lipopolysaccharides. Lytic (virulent) phages infect and lyse the host cell whereas lysogenic (temperate) phages can remain within the host cell with the phage genome incorporated into the host genome. It is very important that only lytic phages are selected for therapeutic use as lysogenic phages do not always lead to host cell death and can facilitate the transfer of potentially harmful genes, for example, those encoding virulence factors and antibiotic resistance into bacterial cells. Interest and research into phage therapies in the West was diminished with the discovery of antibiotics although continued in Eastern Europe especially in Poland and Georgia where well established Phage Institutes continue to this day.

The serious problem of antibiotic resistance in ETEC strains and political and consumer pressure to reduce antibiotic use in food production has led to a revival in interest in phage therapies. Williams Smith and Huggins in the 1970-80s successfully treated diarrhoeal calves, lambs and piglets with phages [3, 4]. More recently Jamalludeen *et al* used a phage cocktail to treat ETEC infected piglets [5, 6]. The use of a mixture of phages in a cocktail reduces the emergence of phage resistant ETEC strains. It is inevitable that resistance will occur however Williams Smith noted reduced pathogenicity in these and ultimately phages effective against the resistant strains can be readily isolated [3].

The aims of this trial were to isolate and characterise bacteriophages specifically lytic for porcine ETEC strains from Corowa and assess the therapeutic efficacy of an orally administered phage cocktail.

2. Methodology

In cooperation with Rivalea a panel of bacteriophages was assessed for efficacy as treatment for ETEC infection in piglets. The phages had been isolated & purified using enrichment protocols with effluent & soiled bedding from modules at Corowa. The aim was to treat three litters at the onset of scouring (treatment group) and compare their progress with three litters that received the standard Rivalea treatment (control group). The standard Rivalea treatment was electrolyte administration via automatic waterer. No antibiotics were used in either control or treatment groups. The trial was carried out in Module 3.

Rectal swabs were collected from all animals on day 0 prior to treatment and on days 2 and 6 after treatment. All samples were held @ 4°C & transported to the Microbiology laboratory at the University of South Australia for processing. Samples were cultured and *E. coli* isolates tested for phage susceptibility. In addition, the swabs were tested for the presence of the bacteriophages. Piglets were monitored daily for any adverse reactions and mortalities recorded. Days 0, 2 and 6 all piglets were scored for scouring (ss = 0, 1, 2 or 3) using the standard Rivalea protocol and weighed. Environmental samples (pen, feeder, waterer) were collected from both the treatment and control crates and tested for the presence of bacteriophages. All swabs were stored in Stuarts transport medium. Piglets treated with the bacteriophage cocktail were euthanized at weaning to comply with APVMA requirements and disposed of by deep burial.

1. **Environmental samples**
    a. Swabs of the six pens housing the test & control piglets were taken on days 0, 2 and 6.
2. **Treatment protocol:**
    a. **Day 0, 8:30 am**: Rectal swabs of test (28) & control (29) piglets were taken.

    b. Piglets in treatment groups; 10 min prior to phage treatment each piglet was orally dosed with 1 mL of the commercial product Gastrogel antacid treatment (2 mL= aluminium hydroxide 100 mg, Mg trisilicate 48 mg, Mg hydroxide 48 mg).

    c. Piglets were orally dosed with 3 mL of the phage cocktail suspended in skim milk.

    d. **Day 0, 3 pm**: Piglets in treatment groups were dosed with Gastrogel & phage cocktail as above.

The phage cocktail consisted of a suspension of the phages listed on the table below in SM buffer (Tris-HCl 7.88 g/L, sodium chloride 5.85 g/L, magnesium sulphate 1.97 g/L, gelatine 0.1 g/L, pH 7.4 – 7.5) with skim milk:

Phage ID	Titre pfu/mL*	Phage ID	Titre pfu/mL*	Phage ID	Titre pfu/mL*
C11	1.2×10^{8}	**C47**	1.9×10^{9}	**C55**	2.0×10^{9}
C13	4.3×10^{8}	**C48**	3.4×10^{9}	**C56**	2.5×10^{9}
C27	1.3×10^{8}	**C49**	5.1×10^{9}	**C57**	6.9×10^{8}
C34	3.2×10^{8}	**C50**	3.7×10^{9}	**C58**	7.4×10^{9}
C42	1.8×10^{8}	**C51**	3.3×10^{9}	**C59**	4.1×10^{8}
C43	3.8×10^{9}	**C52**	3.3×10^{9}	**C60**	6.9×10^{8}
C44	2.8×10^{9}	**C53**	4.9×10^{9}	**C61**	1.2×10^{9}
C45	3.2×10^{9}	**C54**	3.3×10^{9}	**C62**	1.1×10^{9}
C46	1.5×10^{9}				

*pfu/mL = plaque forming units per mL

3. **Sample preparation (Rectal swabs):**
   a. Swabs were streaked on both sheep blood agar (SBA) and MacConkey agar (Mac#3).
   b. Plates were incubated O/N @ 37°C.
4. Two colonies from rectal swabs preferably haemolytic & lactose positive strains were tested as prioritised (A preferred) by the following table:

	Blood agar	MacConkey agar
A	1 haemolytic	1 lactose positive
B	1 non-haemolytic	1 lactose positive

   a. Phage susceptibility was determined using the host range spot test:
      i. The colony was inoculated into LGB & incubated O/N @ 37°C, rotating gently.
      ii. The O/N broth (250 µL) was inoculated into 5mL LGB & incubated for 90 min @ 37°C, rotating gently.
      iii. Overlays were inoculated with 200 µL of log phase *E. coli* & poured onto LGCA plates.
      iv. 2 µL aliquots of each phage were applied in rows.
      v. Plates were dried, inverted & incubated O/N @ 37°C.
      vi. Checked for zones of clearing.

b. Piglets in treatment group Pen 125 were re-tested using a second colony from the SBA day 0 plate & colonies from day 6 as described in 4.a.

c. A number of colonies were selected to check identification by Gram's stain (Gram negative rods) & oxidase test (oxidase negative).

d. Piglet 3212 was sampled from the small intestine post mortem & a colony from the SBA plate tested as described in 4.a.

5. Environmental sampling (swabs):

a. The swab was placed in a tube with 2 mL of SM buffer for phage detection.

6. Phage detection (faecal & environmental swabs):

a. One drop of chloroform was added to the SM buffer suspension.
b. Held at room temperature for 15 min.
c. Centrifuged @ 4000 g for 10 min.
d. The supernatant was filtered through 0.45 µm membrane.
e. 450 µL aliquot of each filtered suspension was mixed with 50 µL of ETEC strain Ec200.
f. Incubated @ 37°C for 60 min.
g. Quadruple 20 µL aliquots were spotted onto LGCA plates.
h. Control plate 20 µL aliquot of Ec200 only was also inoculated.
i. Incubated O/N at 37°C.
j. Checked for the presence of plaques (observed as holes in the bacterial lawn).

7. Statistical analysis

The trial data was analysed by unpaired t-test using GraphPad Prism (GraphPad Software Inc., La Jolla, California, USA). A difference was rated as significant if the P-value was equal to or less than 0.05.

3. Outcomes

Between Jan and August 2012 this laboratory received 19 toxigenic *E. coli* isolates from scouring piglets representing 4 virotypes (as provided by ACE laboratories, Bendigo). The serogroups were not determined. Two strains were non-haemolytic on SBA. In total, 16 phages specific for these ETEC strains were isolated and purified although it is likely that the actual number of different phages is less as some may have been isolated on multiple hosts. Further analysis of the phage genomes by restriction fragment length polymorphism (RFLP) or sequencing would be required to differentiate them.

The phage cocktail was lytic for 11 of the 19 strains. The success of the trial would be dependant on the actual ETEC strains present. It is also likely that the piglets could be infected by more than one strain so even if the phages were able to eliminate or reduce the bacterial load of one pathogen the remainder could proliferate. The data and statistical analysis are detailed in Tables 1 and 2 (Appendices: Tables 1 & 2). The rectal swab results indicate that at least one non-haemolytic ETEC had infected the majority of piglets. Only 4 treatment piglets, all in Pen 125 (total 7), had significant numbers of haemolytic *E. coli*. This indicates they were likely infected with both haemolytic & non-haemolytic ETECs. All the piglets in the litter from Pen 125 were severely undernourished, underweight and lethargic with scour scores of 2 & 3. They were also only 10 days old compared to the remaining litters that ranged from 14 to 18 do. Unfortunately there is no control group with the same pathogen profile. It is not possible to compare the efficacy of the treatment with an untreated group. The smallest piglet had died by day 1. This is unlikely to be related to the treatment as its condition was initially very poor. The post mortem samples showed mixed microflora and no *E. coli* isolates were lysed by the phages. It is worth noting that the remaining piglets in this litter survived and that three piglets with scour scores of 3 and one with a score of 2 were 0 by day 2. It is likely that the phage treatment contributed to the rapid reduction of scouring and survival of these piglets. **The results indicate this group was the only one likely to respond to the phage cocktail and did respond positively especially given their very poor condition.**

No phages were detected in any samples from the control groups. Phages were detected in day 2 samples of 18 piglets in the treatment litters. No phages were detected from days 0 or 6 swabs. For day 2 treatment samples where no phages were detected there are several explanations:

1. Phages didn't survive transit through the stomach although this is unlikely as the administration methodology was shown to be successful in 18 treatment piglets.
2. Phages passed through the GIT & were excreted prior to day 2 sampling.
3. The sample (rectal swab) was an insufficient amount for detection i.e. below the limit of detection.

Phages were detected in environmental day 2 swabs from two treatment pens. ETEC and phage concentrations in the faeces could not be determined with this sampling protocol however actual numbers were not required for the trial.

The phage cocktail was lytic for the haemolytic *E coli* (presumptive ETECs) isolated from the two piglets in pen 125. Testing of more isolates per culture plate from the piglets in this and other pens may well have detected more phage susceptible strains. The day 6 cultures from pen 125 were not susceptible to the phages investigated and this indicates the rapid change in *E coli* strains in this piggery. No isolates tested from the control groups or remaining treatment groups were lysed but again this may reflect that fact that only two single colonies from each piglet were tested. The clinical results suggest that phage may have been effective in treating the piglets in pen 125 but the laboratory data to support this conclusion could be stronger.

The multifactorial nature of ND and PWD is highlighted by the changes in management in module 3 that have reduced the incidence of both. Modifications include a significant reduction in agalactia in sows using step up feeding: start feed of 1 Kg per day on the day of birth then step up to ad lib by 4 days. Overfeeding sows during gestation and lactation can lead to agalactia especially in those with particular genetic profiles. The low protein weaner diet and modified cleaning procedures using 'Black extreme' disinfectant are also factors contributing to the reduction in incidence and severity of infections.

Table 3 in the appendices is a summary of ETEC isolates from Corowa with two or more genes for virulence factors as determined by PCR at UniSA and Ace laboratories, Bendigo. It is evident from this data that for the years 2008 to 2009 the virulence profile and serogroups of isolates remained relatively consistent. No strains from 2010 were tested. In 2011 a transition occurred and different strains became apparent as the predominant cause of diarrhoea until mid 2012. The table clearly highlights the extreme diversity of ETEC isolates that have been detected since July 2012. It is likely that the controls implemented have suppressed the prevalent strains and these previously less common ETECs are emerging or have evolved.

4. Application of Research

Successful treatment of ETEC infection using bacteriophage therapy could improve productivity by limiting losses due to reduction in meat production, mortality and treatment costs. The high level of antibiotic resistance in these ETECs limits treatment options and phages are effective against such strains. Phages are cheaper to produce than antibiotics but it is critical that they are lytic. A phage library or collection should be developed. The ETEC strains would be screened against available phages to identify suitable isolates for treatment. If necessary phages specific for the ETEC strains could be readily isolated, characterised and added to the library. The efficacy of the treatment strategy has been shown to be promising in this trial but the results were not definitive and highlight the importance of building on to the existing collection of lytic phages specific for ETECs.

5. Conclusion

Rivalea at Corowa has multiple haemolytic and non-haemolytic toxigenic strains of *E. coli*. These are differentiated on the basis of virulence factor profiles determined by PCR and haemolytic status on sheep blood agar. Historically the strains of ETEC isolated from Australian piggeries have remained relatively stable & consistent with haemolytic O149 F4:STa:STb:LT the most prevalent. PFGE analysis has shown that O149 ETEC isolates from different geographical regions tend to be highly clonal [7, 8]. Smith *et al* have shown that strain and plasmid diversity reflects selection pressures at individual farm level [9]. The Corowa facility appears to have greater diversity for an individual site than reported in previous studies. Determination of the selection pressures leading to emergence

of multiple virotypes will be critical for understanding the causes of the rapid emergence of new ETEC strains.
The bacteriophages isolated have been shown to be highly specific for their host strains. This is desirable as it limits collateral damage to non-pathogenic or commensal bacterial types present in the GIT of the piglets. The limitation is that with so many potential pathogens present in the farrowing sheds and such a dynamic bacterial environment it is difficult to formulate a phage cocktail that will cover all the possible pathogenic strains. Of significance is that it was not known prior to the trial which ETECs were infecting the piglets. The results indicate multiple strains were present. There were no phages in the cocktail that would lyse the majority of strains. The one litter that was infected with ETECs that could be treated with the phage cocktail did respond positively. Further enrichment & isolation using the other ETECs present would need to be carried out to formulate a cocktail with the potential to treat the remaining litters.

6. Limitations/Risks

Bacteriophages must be specific for the ETEC strains present. There is no therapeutic value if the pathogenic *E. coli* are not lysed by the phages. The phages must be assessed as lytic (virulent) not lysogenic (temperate) for the strains present. The risk would be incomplete death of the target bacteria & the potential transfer of undesirable genes, for example, antibiotic resistance or virulence factor genes, to other bacterial cells.

7. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

1. A trial should be carried out in a facility with a limited number of known strains of ETEC, that is, with a more stable profile of potential pathogenic strains.
2. Wherever feasible, the identification of the ETEC strains present should be determined prior to administration of the phage cocktail.
3. Isolation & characterisation of lytic phages specific for ETECs, particularly emerging strains, should continue to add potential therapeutic candidates to the phage library.

8. References

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Appendices:

Table 1: Individual piglet data

SHED NO.	PEN NO.	SOW ID	PARITY AT ENTRY TO FH	Group	DOB	Piglet ID	Sex	Day 0 weight Kg 18/9/12	day 0 scour score	day 2 weight Kg 20/9/12	day 2 scour score	day 6 weight Kg 24/9/12	day 6 scour score	ADG	Average ADG
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3257	F	3.3	1	3.5	0	5	0	0.3	
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3258	F	4.4	1	4.8	0	5.9	0	0.3	
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3259	M	4.9	1	5.2	0	6.7	0	0.3	
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3260	F	4.8	1	4.6	0	6.3	0	0.3	
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3261	F	5	1	5.2	1	7.1	0	0.4	
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3262	F	4.8	1	5.1	3	6.9	0	0.4	
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3263	M	5.1	1	5.8	1	7.8	0	0.5	
mod 3 shed 2	26	4D1305	1	control	3/09/2012	3264	F	4.6	2	4.5	3	5.6	0	0.2	0.3
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3265	M	3	1	3.3	0	4.5	0	0.3	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3266	M	2.8	3	3	0	3.8	0	0.2	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3267	M	2.3	1	2.5	1	3.6	1	0.2	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3268	F	2.2	0	2.3	1	3	0	0.1	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3269	F	2.6	1	2.8	0	3.5	0	0.2	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3270	F	2.4	3	2.4	2	3.1	0	0.1	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3271	M	2.6	3	2.7	2	3.2	0	0.1	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3272	F	3	0	2.3	1	4.6	0	0.3	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3273	F	2.1	1	2.2	0	3	0	0.2	
mod 3 shed 2	116	3D5448	0	control	4/09/2012	3274	M	2.9	1	3	0	4.2	0	0.2	0.2
mod 3 shed 2	107	3E3281	0	control	4/09/2012	3275	M	4.3	1	4.3	1	4.7	0	0.1	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3276	F	3.3	1	3	1	3.4	0	0.02	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3277	M	4	2	4.3	0	5.5	0	0.3	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3278	M	4.2	1	3.9	0	4.6	0	0.1	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3279	M	4.3	0	4.5	0	5.8	0	0.3	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3280	M	2.4	0	2.6	0	2.8	0	0.1	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3281	F	4	0	3.9	0	3.9	1	-0.02	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3282	F	4.2	0	4.5	1	5	1	0.1	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3283	F	4.4	0	4.9	0	5.6	1	0.2	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3284	M	4	1	4.4	0	5.2	0	0.2	
mod 3 shed 2	107	3D5448	0	control	4/09/2012	3285	F	4.5	0	4.4	1	4.4	0	-0.02	0.1
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3201	M	3.7	0	4.2	1	5.5	0	0.3	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3202	M	4	3	4.2	3	5.2	0	0.2	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3203	F	3.8	0	4.2	1	5.3	0	0.3	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3204	M	3.9	0	3.9	0	4.8	0	0.2	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3205	M	4	1	4.5	1	5.6	0	0.3	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3206	F	4.3	3	4.2	2	5.5	0	0.2	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3207	F	4.5	0	4.8	0	6	0	0.3	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3208	F	3.4	0/blood	3.8	0	4.9	0	0.3	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3209	M	5	1	5.9	2	7	0	0.3	
mod 3 shed 2	104	3D6361	0	treatment	3/09/2012	3210	F	4.4	2	5.2	2	6.7	0	0.4	0.3
mod 3 shed 2	125	3D9451	0	treatment	8/09/2012	3211	F	1.4	3	1.5	0	1.8	0	0.1	
mod 3 shed 2	125	3D9451	0	treatment	8/09/2012	3212	F	1.2	3	X	X	X	X	X	
mod 3 shed 2	125	3D9451	0	treatment	8/09/2012	3213	F	2.3	2	2.4	0	2.6	1	0.1	
mod 3 shed 2	125	3D9451	0	treatment	8/09/2012	3214	M	2	2	2.2	2	2.5	1	0.1	
mod 3 shed 2	125	3D9451	0	treatment	8/09/2012	3215	M	1.7	3	1.6	3	1.9	0	0.03	
mod 3 shed 2	125	3D9451	0	treatment	8/09/2012	3216	M	2.1	3	2.2	0	2.1	1	0.0	
mod 3 shed 2	125	3D9451	0	treatment	8/09/2012	3217	F	2.7	3	2.7	0	3.2	0	0.1	0.1
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3218	F	5.7	0	6.6	0	7	0	0.2	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3219	F	5.1	1	5.7	0	6.4	0	0.2	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3220	F	5.8	0	6.9	0	7.4	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3221	F	5.6	3	6	0	7.6	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3222	M	4.8	3	5.6	1	6.5	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3223	F	2.8	1	3.3	0	4.7	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3224	F	4.1	1	4.6	0	6.1	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3225	F	4.7	0	5	0	6.7	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3226	F	6.3	0	7.2	0	8.3	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3227	M	5.9	3	6.4	0	7.7	0	0.3	
mod 3 shed 2	3	3A4895	3	treatment	31/08/2012	3228	M	3.5	2	4.1	1	4.8	0	0.2	0.3
P-value									0.0797		0.8484		0.7668		0.4277
Significance									none		none		none		none

Table 2: Individual piglet data cont.

SHED NO.	PEN NO.	Group	Piglet ID	β-haem *E. coli*	faecal phage	environ phage	β-haem *E. coli*	faecal phage	environ phage	β-haem *E. coli*	faecal phage	environ phage
				day 0			day 2			day 6		
mod 3 shed 2	26	control	3257	0	0	0	0	0	0	0	0	0
mod 3 shed 2	26	control	3258	0	0		0	0		0	0	
mod 3 shed 2	26	control	3259	0	0		0	0		0	0	
mod 3 shed 2	26	control	3260	0	0		0	0		0	0	
mod 3 shed 2	26	control	3261	0	0		0	0		0	0	
mod 3 shed 2	26	control	3262	0	0		0	0		0	0	
mod 3 shed 2	26	control	3263	0	0		0	0		0	0	
mod 3 shed 2	26	control	3264	0	0		0	0		0	0	
mod 3 shed 2	116	control	3265	0	0	0	0	0	0	0	0	0
mod 3 shed 2	116	control	3266	0	0		0	0		0	0	
mod 3 shed 2	116	control	3267	0	0		0	0		0	0	
mod 3 shed 2	116	control	3268	0	0		0	0		0	0	
mod 3 shed 2	116	control	3269	0	0		0	0		0	0	
mod 3 shed 2	116	control	3270	0	0		0	0		0	0	
mod 3 shed 2	116	control	3271	0	0		0	0		0	0	
mod 3 shed 2	116	control	3272	0	0		0	0		0	0	
mod 3 shed 2	116	control	3273	0	0		0	0		0	0	
mod 3 shed 2	116	control	3274	0	0		0	0		0	0	
mod 3 shed 2	107	control	3275	0	0	0	0	0	0	0	0	0
mod 3 shed 2	107	control	3276	0	0		0	0		0	0	
mod 3 shed 2	107	control	3277	0	0		0	0		0	0	
mod 3 shed 2	107	control	3278	0	0		0	0		0	0	
mod 3 shed 2	107	control	3279	0	0		0	0		0	0	
mod 3 shed 2	107	control	3280	0	0		0	0		0	0	
mod 3 shed 2	107	control	3281	0	0		0	0		0	0	
mod 3 shed 2	107	control	3282	0	0		0	0		0	0	
mod 3 shed 2	107	control	3283	0	0		0	0		0	0	
mod 3 shed 2	107	control	3284	0	0		0	0		0	0	
mod 3 shed 2	107	control	3285	0	0		0	0		0	0	
mod 3 shed 2	104	treatment	3201	0	0	0	0	✓	0	0	0	0
mod 3 shed 2	104	treatment	3202	0	0		0	✓		0	0	
mod 3 shed 2	104	treatment	3203	0	0		0	✓		0	0	
mod 3 shed 2	104	treatment	3204	0	0		0	✓		0	0	
mod 3 shed 2	104	treatment	3205	0	0		0	0		0	0	
mod 3 shed 2	104	treatment	3206	0	0		0	✓		0	0	
mod 3 shed 2	104	treatment	3207	0	0		0	0		0	0	
mod 3 shed 2	104	treatment	3208	0	0		0	✓		0	0	
mod 3 shed 2	104	treatment	3209	0	0		0	0		0	0	
mod 3 shed 2	104	treatment	3210	0	0		0	✓		0	0	
mod 3 shed 2	125	treatment	3211	0	0	0	✓	0	✓	0	0	0
mod 3 shed 2	125	treatment	3212	0	0		0	✓		0	0	
mod 3 shed 2	125	treatment	3213	✓	0		✓	✓		✓	0	
mod 3 shed 2	125	treatment	3214	✓	0		✓	✓		✓	0	
mod 3 shed 2	125	treatment	3215	0	0		0	✓		0	0	
mod 3 shed 2	125	treatment	3216	✓	0		✓	✓		✓	0	
mod 3 shed 2	125	treatment	3217	✓	0		✓	✓		✓	0	
mod 3 shed 2	3	treatment	3218	0	0	0	0	0	✓	0	0	0
mod 3 shed 2	3	treatment	3219	0	0		0	✓		0	0	
mod 3 shed 2	3	treatment	3220	0	0		0	0		0	0	
mod 3 shed 2	3	treatment	3221	0	0		0	0		0	0	
mod 3 shed 2	3	treatment	3222	0	0		0	✓		0	0	
mod 3 shed 2	3	treatment	3223	0	0		0	0		0	0	
mod 3 shed 2	3	treatment	3224	0	0		0	0		0	0	
mod 3 shed 2	3	treatment	3225	0	0		0	0		0	0	
mod 3 shed 2	3	treatment	3226	0	0		0	0		0	0	
mod 3 shed 2	3	treatment	3227	0	0		0	✓		0	0	
mod 3 shed 2	3	treatment	3228	0	0		0	✓		0	0	
mod 3 shed 2	3	treatment	3228	0	0		0	✓		0	0	

Table 3: ETEC isolates from Corowa 2008 to 2012

Date collected	Serotype	β-haem	Virulence factor genes by PCR												
			F4	F5	F6	F18	F41	LT	STa	STb	Stx2e	EAST1	Paa	SepA	eaeA
2008	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
x.02.09	NRS	+	+	-	-	-	-	+	+	+	-	+	-	+	nd*
18.11.09	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	NRS	+	+	-	-	-	-	-	+	+	-	-	+	-	nd
"	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	NRS	+	+	-	-	-	-	-	+	+	-	-	-	-	nd
"	O157	+	+	-	-	-	-	+	+	+	-	+	+	-	-
"	O:98	+	-	-	-	-	-	-	-	-	-	+	+	-	-
"	O:101	-	-	-	-	-	-	-	+	-	-	+	-	-	-
"	NRS	+	-	-	-	-	-	-	-	-	-	+	-	-	-
"	NRS	+	-	-	-	-	-	-	-	-	-	+	-	-	-
10.05.11	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
11.05.11	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
19.05.11	O157	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	O157	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
x.x.11	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
17.08.11	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
21.03.12	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
11.04.12	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
26.04.12	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
21.05.12	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
27.06.12	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
19.07.12	nd	-	-	-	-	-	-	+	-	+	-	+	nd	nd	-
"	nd	-	-	+	-	-	-	-	+	-	-	+	nd	nd	-
27.06.12	nd	+	+	-	-	-	-	+	+	+	-	+	nd	nd	-
"	nd	+	+	-	-	-	-	+	+	+	-	+	nd	nd	-
19.07.12	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	+	+	-	-	-	-	+	+	+	-	+	nd	nd	-
22.08.12	nd	-	-	-	-	-	-	-	-	+	-	+	nd	nd	-
"	nd	+	+	-	-	-	-	+	+	+	-	+	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	-	-	-	-	-	-	+	-	+	-	+	nd	nd	-
29.08.12	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-
"	nd	-	-	-	-	-	-	-	-	+	-	+	nd	nd	-
"	nd	+	+	-	-	-	-	-	+	+	-	-	nd	nd	-

*nd = not determined