

Research and Development

Final Project Report

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Project title

Transfer of antimicrobial resistance genes between bacteria in stored and spread farm wastes

DEFRA project code

OD2008

Contractor organisation and location

Veterinary Laboratories Agency, Woodham Lane, New Haw, ADDLESTONE, Surrey

Total DEFRA project costs

£ 386,957

Project start date

01/09/00

Project end date

31/08/03

Executive summary (maximum 2 sides A4)

Main objectives

- 01 - Assessment of transfer of resistance between organisms in stored farm wastes
- 02 - Assessment of transfer of resistance in spread farm wastes
- 03 - Assessment of transfer of resistance in treated farm wastes
- 04 - Assessment of genetic basis of acquired resistance in marked organisms
- 05 - Development of models for transfer of resistance

Methods used

Relevant databases and personal contacts were utilised to assemble a panel of donor and recipient organisms containing a range of transferable resistance determinants and marker genes. Horizontal gene transfer experiments (conjugation, transformation) were carried out in a variety of laboratory and field models to determine the risk of transfer of antimicrobial resistance in animal waste during storage and after spreading onto grazing fields. Methods for sample waste collection and for setting large-scale models under biosecure conditions are provided in the report. Transconjugant organisms were selected on antibiotic containing plates with the correct combinations to allow only recovery of marked recipient organisms. Genetic characterisation of the organisms was performed by PCR for antimicrobial resistance determinants, pulsed field gel electrophoresis, and plasmid profiling. A longitudinal storage model was used as a treatment strategy. Finally deterministic and stochastic risk models have been developed and reviewed by the Modeling Team.

Main findings of the research

Storage models

No conjugation occurred for all the organisms tested, under all the experimental conditions described within poultry, cattle and pig faecal waste laboratory storage models. No difference was found between sterile and untreated fresh waste

models, which implies that chemical composition and nutrient deficient conditions may be the cause of inhibition rather than competition by resident flora.

Similarly, there was no conjugation for all the organisms tested, under all the experimental conditions described within cattle and pig farm-scale storage models. In the case of the poultry farm-scale models, conjugation was detected after 5 and 24 h, however the organisms were non-detectable after 48 h probably due to the rapid increase of the temperature in the manure heaps.

Spreading models.

Conjugation was detected after 24 h and up to 7 days in the laboratory models simulating spread of all 3 waste types.

No conjugation was detected from the field-scale model experiments in March, however the second experiments in June did show that conjugation was possible. This difference may imply that temperature as well as other climatic conditions may influence the outcome of conjugation in field conditions. We may conclude that warmer temperatures in the summer season favoured the transfer of the resistance determinants by conjugation..

In summary, results from our experiments suggest that stored slurry is not a good environment for conjugation. However, when applied to pasture, animal manure could provide a favourable environment for exchange of genetic material between bacteria, facilitating the spread of antimicrobial resistance.

Studies on transfer of naked DNA.

The naked DNA experiments have not yielded positive results under the experimental conditions so far employed. We therefore consider that on the basis of results so far achieved, the uptake of naked DNA by pathogenic bacteria in stored organic wastes is unlikely. Annex 10 provides the full details on these experiments.

Treatment models.

Initially, models simulating stirring tanks, anaerobic or aerobic digestion, lime addition were thought to be adequate for this section of the project. However, most farms would struggle to finance treatments of this sort. Most farms only have 1 slurry store and this store is added to on a continual basis. As a consequence a more realistic approach was followed. Storage without extra additions is the cheapest and most pragmatic way to treat pathogen-containing manures. What we have done is to assess the effectiveness of a very cheap treatment method for manures by following long-term storage up to 96 days. In spite of the presence of “natural” transferable elements in the waste, and of the external source of donor strains added in the model, no transfer of resistance determinants to our recipient strains could be detected.

Risk-assessment models.

Using the data from the experimental work carried out at the Veterinary Laboratories Agency and the Health Protection Agency, risk assessment models were developed to estimate the risk of antimicrobial resistance gene transfer in stored and spread farm waste. Each model describes the storage and spreading practices of farm waste and investigates the impact of such practices on the transfer of antimicrobial resistance genes. The models predict that the risk of antimicrobial resistance gene transfer in stored and spread waste is low, and that the risk to livestock grazing on land spread with farm waste is equally low. However, the models indicated that this risk could be reduced further by, for example, increasing the time between the last spreading of waste and the time of turnout. A separate report provides the full details of the risk assessment models

Prime options for new work.

Having demonstrated some risk in association with spread waste a wider survey of the potential of recipient organisms to acquire resistance from freshly spread waste and waste that has been left for the recommended period before grazing. The risk of uptake of such organisms by wildlife and contamination of salad crops should also be determined. The question remains what is the risk for transfer of resistance once the organisms have been acquired by grazing animals in the field. A collaborative project with Bristol University has been proposed looking at transfer or resistance genes in *Salmonella* in vivo pig models. The experience gathered using the strains in OD2008 will be invaluable for the new proposed work. Similar controlled studies should also be carried out in grazing animals. Additional work should also be carried out to determine whether antimicrobial pollution in the environment, particularly in conditions simulating aquaculture in the Far East, may enhance transfer of antimicrobial resistance and linked virulence genes.

Scientific report (maximum 20 sides A4)**BACKGROUND TO THE WORK**

A full description is provided in Annex 1.

AIMS OF THE PROJECT

The purpose of this project has been to provide an assessment of the potential for transfer of antibiotic resistances between bacteria present in farm organic wastes when the wastes are stored and spread on to land. Storage and spreading laboratory and farm models have been set up for a variety of wastes from 3 different animal species (cattle, pig and poultry). The data obtained provides information on dissemination of antibiotic resistance determinants through agricultural practices, also the project has generated a risk assessment to evaluate the potential risk of animal waste (slurry, manure) as a factor which may enable the spread of antibiotic resistance genes.

This work has concentrated on organisms which occur in organic wastes and are of major current concern in relation to antimicrobial resistance: *Salmonella typhimurium*, commensal *E.coli*, and *Enterococcus faecium*. All of these organisms have been included in the work planned in this project. Sensitive strains of these organisms marked genetically to facilitate their retrieval from the waste were used as recipients. Genetic techniques have been used to confirm the transfer of genes between bacterial strains. The transfer of naked DNA, which survives the death of organisms in stored and treated farm wastes has also been studied.

The information provided in the background section summarises a variety of research work which has been published to study gene transfer in organic waste and soil. Transfer has been demonstrated to be able to occur by several means: conjugation, transformation and transduction. The majority of the experiments conducted in the past used what may be termed "worst case" scenarios, in that transfer was encouraged through the use of well established donor and recipient strains, or transforming DNA. It may be considered, that if transfer is not observed under proven favorable conditions, then it is unlikely to take place in the field where the pertaining conditions may be otherwise.

The data generated from the laboratory models has provided basic information about whether storage or spreading wastes containing antibiotic resistant bacteria can influence transfer of these resistances. This information has subsequently been tested in field studies. These studies have comprised storage of waste at the farm, and spread of waste onto pasture. The data generated in the field studies has served to validate that produced in the laboratory models.

In addition the work has enabled the VLA to develop closer working relationships with HPA (formerly CPHL) and Direct Laboratories (formerly ADAS) and to harmonise methodologies for the investigation of antimicrobial resistance genes. The findings from this research will assist DEFRA in further refining the guidance it provides on the handling of organic wastes so that it takes full account of the risks of antibiotic resistance transfer. Such guidance currently does not take full account of the microbiological issues in particular the dissemination of pathogens and other undesirable organisms in the environment.

OVERALL RESPONSIBILITIES

VLA

laboratory scale models, bacterial isolation and phenotypic resistance characterisation, some genetic confirmation, development of general model frameworks and quantitative risk assessment models. Data from the laboratory and field studies in this proposal will provide input for the risk assessment.

Direct Laboratories (formerly ADAS)

farm scale models, field models and collection of samples and data from the field.

Health Protection Agency (formerly PHLS)

consultancy, donor and recipient isolates, characterisation of transferred resistance genes, isolation, characterization and transfer experiments of naked DNA.

University of Glasgow Veterinary School

Consultancy on risk assessment

ACHIEVEMENT OF OBJECTIVES

OBJECTIVE 1.

Assessment of transfer of resistance between organisms in stored farm wastes

A model for waste storage will be developed at VLA Weybridge and ADAS. Transfer of resistance from a range of wastes to a range of susceptible marked organisms and the contribution of naked DNA will be studied. Results from the models will be used to design field scale studies.

Planning

Primary milestone (01/01). A planning meeting of collaborators was organized on 05/06/00 and discussions took place around critical issues such as origin and selection of organisms for the study, construction of laboratory models, and protocols for conjugation studies. Minutes are attached (Annex 2).

Donor and Recipient organisms

Primary Milestone (01/02). Relevant databases and personal contacts were utilised to assemble a panel of donor and recipient organisms containing a range of transferable resistance determinants and marker genes.

Salmonella Typhimurium P5280407 phage-type DT204b originating from a recent international outbreak (37) was used as donor. This strain was resistant to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulphonamides, tetracyclines, trimethoprim, nalidixic acid, and presented decreased susceptibility to ciprofloxacin (R-type ACGKSSuTTmNx_{Cp}_I). The strain harbours a plasmid of 120 MDa responsible for the ACGKSSuTTm phenotype belonging to plasmid incompatibility group H2.

Escherichia coli K12 14R525 resistant to nalidixic acid and carrying a 120 MDa plasmid resulting from conjugation with *S. Typhimurium* P528040 was used as donor.

Two plasmid free strains, *Escherichia coli* K12 20R764 and *Salmonella* Typhimurium 26R755 DT36, both rifampicin resistant were used as recipients in the conjugation experiments.

Enterococcus faecalis NCTC 12201 vancomycin resistant, and *E. faecium* 44Eba tetracycline resistant were used as donors. *E. faecalis* JH2-2 and *E. faecium* G-586, both rifampicin resistant were used as recipients in the experiments. In addition a panel of field and laboratory strains were used as possible donors. A full description of all micro-organisms used in the project is provided in Annex 3.

Culture media.

Antibiotic containing plates were prepared according to standard operating procedures by HPA. Full copies of these documents are provided in Annex 4..

Manures and slurries.

Animal waste was collected following a modified Direct Laboratories protocol. This document is provided in Annex 5. For the purpose of these experiments the term slurry describes a mixture of faecal material, urine, and washing water from intensive systems of management in cattle or pig production. The term poultry manure describes a mixture of faeces originating from a group of birds in intensive production systems. The slurries/manures were obtained from 6 cattle, 6 pig and 6 poultry farms. Bulk samples were collected from the storage tanks and used for the experiments within 24 h of collection.

***In vitro* determination of transfer frequency in broth.**

Starter cultures were prepared with 3 ml Luria Bertani (LB) broth inoculated aerobically with a single colony of the donor or recipient strains and incubated for 24 h at 37°C. 100 ml aliquots of LB broth were seeded with 10⁶/ml donor and 10⁶/ml recipient strains. Transfer of IncH2 plasmids is known to be considerably reduced at temperatures above 34°C. Also, temperatures of 20°C have been found to be more conducive than 37°C to R-plasmid transfer between *E. coli* strains (22). For these reasons, the resultant mixed culture of donor and recipient strains was incubated at room temperature for 5 h. Subsequently, 1 ml aliquots were taken and a series of 10-fold dilutions was prepared with sterile saline solution. Duplicate plates of selective media were inoculated with 0.5 ml of each dilution, and then incubated aerobically for 24 h at 37°C. Recipient counts were obtained by plating 0.5 ml of each dilution on duplicate plates of MacConkey containing rifampicin (100 mg/l). Transconjugants were selected on MacConkey agar containing chloramphenicol (8 mg/l) and rifampicin (100 mg/l). The frequency of plasmid transfer was calculated as the number of transconjugants divided by the number of recipients. To assess whether R-factor transfer would occur on the surface of the medium after seeding, several experiments were conducted in which plates were seeded with broth cultures of donor and recipient strains immediately after they had been mixed, no transconjugants were recovered, these results proved that the conjugation seen in the control experiments had occurred in the broth model and not on the plates. A clear difference in conjugation frequencies was found in the combinations *S. Typhimurium* donor/*E. coli* recipient and *E. coli*

donor/*E. coli* recipient. Data extracted from 34 experiments showed conjugation rates of $18,591 \pm 4,479$ (mean \pm standard error) and of 3.210 ± 654 trans-conjugants / 10^6 recipients respectively.

Survival of donors and recipient in laboratory models.

To check that donors and recipient organisms would survive in the model for the duration of the experiments, 100 ml aliquots of a representative slurry or manure (one for each animal species) were transferred to 200 ml glass bottles and spiked with 10^6 organisms (*S. Typhimurium* donor, *E. coli* donor, or *E. coli* recipient) /ml. These bottles were incubated at 28°C and subsequently sampled 5 h, 24 h, 1, 2 and 3 weeks after inoculation. At sampling time, 1 ml of waste was spread onto duplicate selective plates [MacConkey with rifampicin (100 mg/l) for the recipient strain, or MacConkey with chloramphenicol (8 mg/l) for the donor strains] and then incubated aerobically at 37°C for 24 h. Table 1 summarises the results from the survival experiments. In general terms cattle slurry was the less harmful environment for the micro-organisms, followed by poultry manure and pig slurry. A slight multiplication of the organisms was found in cattle and poultry waste in the first 5 h. In pig slurry after 1 week we could not detect the *E. coli* K12 strains, and after 2 weeks we could not detect the *S. Typhimurium* strain. In the poultry manure model none of the 3 strains were detectable at week 3.

TABLE 1. Survival of donor and recipient organisms in animal waste.

		STM-Donor	EC-Donor	EC-Recipient
Cattle slurry (pH 8)	Time 0	10^{6a}	10^6	10^6
	5 h	$(1.3 \pm 0.4^b) \cdot 10^6$	1.3×10^6	1.8×10^6
	24 h	$(1.4 \pm 0.4) \cdot 10^6$	10^6	5×10^5
	1 w	0.5×10^5	0.42×10^4	4.2×10^4
	2 w	0.7×10^4	10^2	1.5×10^3
	3 w	10^3	10	3×10^2
Pig slurry (pH 8.5)	Time 0	10^6	$10^6/\text{ml}$	$10^6/\text{ml}$
	5 h	$(0.5 \pm 0.2) \cdot 10^6$	1.5×10^5	6×10^5
	24 h	$(0.4 \pm 0.3) \cdot 10^5$	16	7×10^2
	1 w	0.5×10^4	0	0
	2 w	0	0	0
	3 w	0	0	0

Project title	Transfer of antimicrobial resistance genes between bacteria in stored and spread farm wastes	DEFRA project code	OD2008
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	Time 0	10 ⁶ /ml	10 ⁶ /ml	10 ⁶ /ml
	5 h	1.2x10 ⁶	1.1x10 ⁶	1.3x10 ⁶
Poultry manure	24 h	4.4x10 ⁶	11x10 ⁶	4x10 ⁶
(pH 6.5)	1 w	1.3x10 ⁵	1.4x10 ⁵	4.6x10 ⁵
	2 w	6x10 ³	6x10 ⁴	5.5x10 ³
	3 w	0	0	0

^aNumber of cfu / ml of waste

^bResults are presented as mean ± standard error

Study of interaction of donors and recipients in stored sterilised and non-sterilised farm slurry and manure laboratory models.

Primary Milestones (01/03, 01/05). In order to assess the potential for transfer of resistance in artificial models, organisms were inoculated in matched pairs in sterilised and non-sterilised stored waste at ambient and low temperature and the frequency of transfer of resistance assessed by use of selective and non-selective media.

1. Development of a laboratory storage model.

1.1 Sponge chambers

Initially organisms were placed in the waste storage models within sponge chambers. These chambers were constructed by clumping a sponge in a two-piece open-ended plastic vessel suspended on a non-rotting string. Transfer of resistance was assessed in the presence of added donor and recipient organisms. Pig slurry (pH 9.5) was prepared in 3.5 l aliquots in suitable plastic containers and autoclaved 3 times at 121°C for 30 minutes. Sterility was checked before proceeding with the experiments. 10⁹ donor organisms [STM DT204b (ACGKSSuSpTTmNxCPNe) or STM DT104 (ACSSuT)], together with 10⁹ recipient cels (*E. coli* K12 (Rif) were inoculated onto the sponge matrix and plunged into the waste flask. Duplicate waste models were prepared and incubated at 22°C (ambient temperature) and 4°C (low temperature) in temperature controlled rooms. The models were sampled at times 24, 48, 72 hours and 7 days . Inoculation of McConkey selective plates with rifampicin plus one of 11 antibiotics [ampicillin (128 mg/l), chloramphenicol (8 mg/l), gentamicin (32 mg/l), kanamycin (16 mg/l), streptomycin (128 mg/l), sulphathiazole (64 mg/l) , spectinomycin (64 mg/l) , tetracycline (128 mg/l), trimethoprim (2 mg/l), nalidixic acid (16 mg/l), ciprofloxacin (1 mg/l)] did not detect any trans-conjugants in the models.

The same experiment was repeated with non-autoclaved waste with similar results, in this case a separate set of flasks was prepared and inoculated only with recipient organisms to assess the possibility of transfer from the naturally occurring flora, no transconjugants were detected in any of the conditions or combinations tested.

1.2 Nylon filter chambers

As a second attempt to trial chamber devices, a nylon filter chamber model was tested. It consisted of a 6 cm diameter nylon filter membrane attached to a 50 ml capacity vessel. 10^9 donor organisms [STM DT204b (ACGKSSuSpTTmNxCPNe) or STM DT104 (ACSSuT)], together with 10^9 recipient cels (*E. coli* K12 (Rif) were inoculated onto the surface of the filter, then the vessel was filled up with 50 ml of liquefied poultry manure (1:1 in water) and incubated for 5 and 24 h at 37°C. After this incubation time filters were removed from the vessel and briefly rinsed in sterile PBS. Filters were then plated onto selective media (blood-agar containing rifampicin and chloramphenicol) and incubated overnight at 37°C. The idea of this approach was to sample to entire population in the filter. Unfortunately results were difficult to interpret as the enormous bacterial load on the plates was overcoming the inhibitory action of the antibiotics.

1.3 Free mix of organisms

Finally, it was decided that the best approach to allow quantification would be to use a model with free bacteria in the waste model.

The slurry samples came from 6 cattle or pig farms. The pH of the slurry/manure sample was measured on arrival at the laboratory; for the pig waste it was found to vary between 6.5 and 8.5, and for the cattle waste between 7 and 8. On one occasion fresh cattle faeces from an individual animal were collected and used in a single experiment. The poultry manure originated from 6 farms, and bulk samples were collected from the storage heaps and used for the experiments within 24 h of collection. The pH was measured on arrival at the laboratory and was found to vary between 6 and 7.4. Cattle and pig slurries were liquid enough to be used for the experiments with no need for further dilution. However, poultry manure was mixed (1:1 v/v) with sterile distilled water and thoroughly blended using a food mixer prior to the experiments.

1.3.1 Non-sterilised waste

Starter cultures were prepared with 3 ml Luria Bertani (LB) broth inoculated aerobically with a single colony of the donor or recipient strains and incubated for 24 h at 37°C. 100 ml aliquots of waste (cattle, pig, or poultry) were seeded with 10^6 /ml donor and 10^6 /ml recipient strains. The resultant spiked waste sample with donor and recipient strains was incubated at room temperature for 5 h (This was deemed appropriate to reduce the chance of multiplication of transconjugants as well as recipients, which would affect the calculation of the transfer frequency). Thereafter 1 ml aliquots were taken and a series of 10-fold dilutions was prepared with sterile saline solution. Duplicate plates of selective media were inoculated with 0.5 ml of each dilution, and then incubated aerobically for 24 and 48h at 37°C. Recipient counts were obtained by plating 0.5 ml of each dilution on duplicate plates of MacConkey containing rifampicin (100 mg/l). Transconjugants were selected on MacConkey agar containing chloramphenicol (8 mg/l) and rifampicin (100 mg/l). The frequency of plasmid transfer was calculated as the number of transconjugants divided by the number of recipients. In addition, 100 ml aliquots of 9 of the 18 wastes (3 for each animal species) were sterilised by autoclaving twice at 121°C for 15 min; these were tested in parallel to assess the influence of the naturally contaminating flora in the model. All the experiments with each waste (non-sterile and sterile models) were repeated 3 times. No transconjugants

were observed on the selective plates after 24 or 48 h incubation at 37°C for any of the poultry manure samples, or cattle and pig slurries tested.

1.3.2 Sterilised waste

In 3 experiments (per waste type) sterile waste was tested in parallel with the same results (non-detectable conjugation). For every experiment bottles with 100 ml of LB broth were inoculated with donor and recipient organisms as controls to check the viability of the conjugation under ideal conditions.

1.3.3 Extended incubation experiments

A series of experiments was performed according to the protocol described but allowing the models to be incubated for 5, 24, 48 h and 7 days. This was necessary to prove that increasing of contact time between donors and recipients in the models would not result in increased chance of conjugation. A representative waste sample from each of the 3 species was used for these experiments. No conjugation could be detected at any of the sampling times for any of the waste types.

1.3.4 Dilution effects

In order to test the influence of the concentration of slurry on the conjugation process and to assess the level of animal waste necessary to inhibit conjugation, 2 experiments were performed in which the animal waste was diluted. The same model using 100 ml volumes and the previously described conditions were used for these experiments. A series of dilutions (neat manure fresh, neat manure sterile, diluted manure: 1/2, 1/10, diluent only) were prepared in 100 ml volumes and tested with the method previously described. Two diluents were tested in the experiment (sterile distilled water and LB broth). No conjugation was detected in the neat fresh or neat autoclaved waste aliquots. In the first case (water as diluent) no conjugation was detected in any of the dilutions tested; however when LB was used, trans-conjugants were detected at frequencies of $84/10^6$ (1/2 dilution), $1520/10^6$ (1/10 dilution) and $5823/10^6$ (LB broth) for the *S. Typhimurium* donor/*E. coli* recipient combination; and frequencies of $77/10^6$ (1/2 dilution), $1761/10^6$ (1/10 dilution) and $2000/10^6$ (LB broth) for the *E. coli* donor/*E. coli* recipient combination.

2. On farm study of transfer of resistance to recipient organisms in stored wastes (primary milestones 01/07).

Using ADAS contacts, a selection of farm animal wastes were collected and pooled to simulate on farm storage systems. Experiments were conducted for pig and cattle slurry models, poultry manure, and dirty water.

2.1 Microbiology

Recipient and donor strains were cultured for 24h in Luria Bertani broth supplemented with 1.5%(w/v) ammonium chloride. Cultures were grown in 20 l petrol can-style plastic containers without agitation but with an equal volume of media and head space in a CTR at 37°C. After growth, cultures were plated on Luria Bertani Agar to determine levels of each bacterial strain. Cultures were driven from Wolverhampton to Gleadthorpe.

2.2 Tank spiking

For the bovine and pig waste, tanks used were commercially-available farm-scale galvanized iron supports lined with sand and 4mm thick butyl liner. In order to ensure that the tanks were stable and ease sample collection, a JCB was used to create a 1.5m deep trench and the tanks were assembled inside this trench. After assembly, the trench was backfilled. Thus the lower 1.5 m section of the tanks were buried underground.



Tanks were filled with 30,000l of fresh pig or cattle waste.

Prior to addition of bacterial cultures, roughly 40% of the slurry was removed from the tank using a slurry tanker and vacuum pump. The bacterial inoculant solutions were then added to the remaining slurry. The end of the pipe from the slurry tanker was submerged in the slurry remaining in the store. The slurry was emptied back into the store as slowly as possible. In order to effect mixing the waste was slowly pumped back and forth between the store and the tanker. This method avoided splashing and prevented the generation of vacuum or pressures likely to injure the bacteria.



Samples (SOP MICRO-150, Annex 5) were removed from the tanks on days 0, 1, 2, 4, 8, 16, 32 days and sent to VLA for analyses.

2.3 Heap spiking:

Heaps were assembled on a concrete pad in custom built composting bays. Waste liquor (containing bacterial strains) drained into underground collection tanks. Roughly 10 m³ of poultry waste material was used for each experiment. Material was delivered and stacked into each the bay using a JCB. After each load was delivered, a watering can was used to apply either donor or recipient bacteria to the surface of the waste heap.



JCB collecting poultry waste



Empty bays showing drainage system and underground drainage tanks in the background.



Assembling heaps and using a watering can to apply diluted donor/recipient into the heaps.

Samples (SOP MICRO-150, Annex 5) were removed from the tanks on days 0, 1, 2, 4, 8, 16, 32 days and sent to VLA for analyses.

2.4 Pig slurry models.

Prior to tank loading an aliquot of the slurry was sent to the laboratory and screened for the presence of multiresistant *E. coli* by plating on ChromoAgar ECC containing kanamycin (16 mg/l), gentamicin (4 mg/l), chloramphenicol (8 mg/l) and cycloheximide (100 mg/l). The aim of this initial screening was to determine that the slurry used was free of strains with the same resistance phenotype as the experimental donor strain. A storage tank was filled up with 30,000 l of pig slurry originating from several pig farms.

The tank was inoculated with the *S. Typhimurium* donor strain (*S. Typhimurium* P5280407 phage-type DT204b) at 10^7 cfu/ml and samples taken on times 5h, 1, 2, 4, 8, and 14 days. On arrival to the laboratory samples were ten-fold diluted in sterile saline solution and 0.5 ml was plated on the selective ChromoAgar ECC plates and incubated aerobically for 24 h to detect transconjugant *E. coli* strains (defined as blue colonies on the chromogenic media). No trans-conjugants were detected at any of the time points. The first and last sample were also plated on non-selective ChromoAgar ECC plates to estimate the number of *E. coli* in the sample that could act as potential recipient, and the levels were always around 4×10^3 organisms / ml of waste. On day 14, counts for the *S. Typhimurium* donor strains were calculated in the sample (10^7 cfu/ml) and the tank was spiked with $2 \cdot 10^7$ cfu /ml of a marked *E. coli* recipient strain 20R764 (Rif). Samples were taken on days 0, 1, 2, 3, 7 and 14 after inoculation, and were processed as described previously to detect conjugation in the waste. No trans-conjugants were detected on selective plates (ChromoAgar ECC with rifampicin, chloramphenicol, gentamicin and cyclohexamide). At the end of the experiment (day 30) the counts for donor and recipient organisms were calculated and estimated in 10^6 cfu/ml and 0.4×10^6 cfu/ml respectively.

2.5 Poultry storage models.

Poultry manure heaps were used in similar experiments, heaps were prepared in bio-secure conditions at one of the ADAS's sites as described previously.

Poultry manure heap storage model



Manure heaps were inoculated with approximately 10^6 donors and 10^6 recipients per gram of waste. Two separate heaps were prepared and inoculated with the combinations: *S. Typhimurium* donor DT204b (ACGKSSuSpTTmNx CpNe) + *S. Typhimurium* recipient 926R755 DT36 (Rif) and *S. Typhimurium* donor DT204b (ACGKSSuSpTTmNx CpNe) + *E. coli* recipient K12 20R764 (Rif). One hundred gram samples composed of 25 pinches from different locations and depths within the heap were collected at time points 5h, 1, 2, 4, 8, 16 and 32 days. The sample was mixed with the smallest possible amount of sterile distilled water, and this was considered as the neat sample that subsequently was diluted down to 10^{-5} and inoculated on selective plates. Results showed that transconjugants were

selected on plates after 5 h at a rate of 2.3×10^{-3} cnj/rec (ECR) and 4.9×10^{-3} cnj/rec (STMR); and after 24 h at a rate of 3.4×10^{-3} cnj/rec (ECR) and 0.023 cnj/rec (STMR). No recipients or transconjugants were found after 48 h and up to 32 days.

2.6 Cattle storage models.

One farm storage tank was filled up with 30,000 l of cattle beef slurry, and another one with 30,000 l of dirty water from washing yards etc originating from several cattle farms. Both tanks were spiked with *S. Typhimurium* donor strain (DT204b (ACGKSSuSpTTmNx CpNe)] and *S. Typhimurium* recipient (26R755 DT36 Rif^R) at approximately 10^6 cfu/ml and samples taken on times 5h, 1, 2, 4, 8, 16 and 32 days. On arrival at the laboratory samples were ten-fold diluted in sterile saline solution and 0.5 ml was plated on the selective ChromoAgar ECC plates (containing 100 mg/l rifampicin, 8 mg/l chloramphenicol and 200 mg/l cycloheximide) and incubated aerobically for 24 h to detect transconjugant *salmonella* strains. No trans-conjugants were detected on selective plates for any of the sampling times.

OBJECTIVE 02

Study of interaction of donor and recipient organisms in spread farm wastes (primary milestones 02/02, 02/04, 02/06)

1. Culture media.

Antibiotic containing plates were prepared according to standard operating procedures by CPHL. Full copies of these documents are provided in appendix 2.

2. Manures and slurries.

Animal waste was collected following a modified ADAS protocol. This document is provided in appendix 3. For the purpose of these experiments the term slurry describes a mixture of faecal material, urine, and washing water from intensive systems of management in cattle or pig production. The term poultry manure describes a mixture of faeces originating from a group of birds in intensive production systems. The slurries/manures were obtained from 1 cattle, 1 pig and 1 poultry farms. Bulk samples were collected from the storage tanks and used for the experiments within 24 h of collection.

3. Laboratory scale models

Grass models consisting of 800 cm² turf portions were fertilised (50 m³/Ha) with waste (pig, poultry and cattle) containing 10^6 donor and 10^6 recipients/ml and incubated at RT. Samples (30 g) were taken at time 5, 24 and 48 h, 7 and 14 days and dilutions prepared and plated onto selective media. Conjugation was detected after 24 h in the models simulating spread of all 3 waste types. Tables 1, 2 and 3 show a summary of results from laboratory scale experiments. Figure 4 shows an example of transconjugants on selective plates. The presence of the plasmid and the assessment of the PFGE fingerprint proved that these were recipients that had acquired the resistance genetic determinants (this topic will be covered in more detail under objective 4).

Table. 2 Assessment of transfer in spread farm wastes (pig slurry)

TIME	STMD/ECR	STMD/STMR
5 hours	0	0
24 hours	1/377 ^a , 91 ^b	1/80 ^a , 755 ^b
48 hours	1/17 ^a , 2379 ^b	1/101 ^a , 910 ^b
7 days	1/345 ^a , 11 ^b	1/219 ^a , 57 ^b
14 days	6 ^b	1/206 ^a , 30 ^b

^aNumber of transconjugants/number of recipients. ^bNumber of transconjugants/gram of pasture sample.

Table. 3 Assessment of transfer in spread farm wastes (poultry manure)

TIME	STMD/ECR	STMD/STMR
5 hours	0	0
24 hours	1/5200 ^a , 20 ^b	1/1208 ^a , 96 ^b
48 hours	1/34600 ^a , 2 ^b	1/152 ^a , 506 ^b
7 days	0	1/124 ^a , 25 ^b
14 days	0	0

^aNumber of transconjugants/number of recipients. ^bNumber of transconjugants/gram of pasture sample.

Table. 4 Assessment of transfer in spread farm wastes (cattle slurry)

TIME	STMD/ECR	STMD/STMR
5 hours	0	0
24 hours	1/1710 ^a , 70 ^b	1/7857 ^a , 14 ^b
48 hours	1/844 ^a , 50 ^b	1/729 ^a , 51 ^b
7 days	0	1/166 ^a , 24 ^b
14 days	0	0

^aNumber of transconjugants/number of recipients. ^bNumber of transconjugants/gram of pasture sample.

E. coli transconjugants from laboratory turf models.



4. Biosecure pasture plot models

Field-scale models were developed and the transfer of resistance from inoculated donor organisms to recipients in spread farm slurries and manures was assessed over time in different climatic conditions (end of winter March 2003, early summer June 2003).

4.1 Microbiology:

E. coli recipient and *S. Typhimurium* donors and recipients were cultured for 24h in Luria Bertani broth supplemented with 2%(w/v) ammonium chloride. Cultures were grown without agitation but with an equal volume of media and head space. Cultures were plated on Luria Bertani Agar to determine levels of each bacterial strain. Cultures were driven from Wolverhampton to Gleadthorpe.

4.2 Plot preparation:

6 plots of area 3 m² were marked on grass pasture that had not housed stock for >6 months. The plots were secured against scavenging by wildlife by the construction of a wooden frame from (15cm depth x 2.5 cm width) wooden batons. The frames were covered in chicken coup wire mesh (1 cm aperture). An image of the plots is presented in the next figure .

Biosecure plots

Fig. cattle and chicken manures were sourced from local commercial livestock farms. 25 l or 25 kg of material was spread onto each plot (a water control was used for the pilot study). 25 ml of either *E. coli* or *S. Typhimurium* donor was added to each manure. An equal volume of recipient culture was also added. The manure and cultures were mixed by closing the drum containing the manure and rolling it back and forth for 5 min. Material was then spread evenly into the pasture plots. The chicken wire was nailed into the wooded frames to prevent scavenging and infection of wildlife.

4.3 Sample frequency and analysis:

First samples were taken 5 h after manure spreading. Plugs of pasture (10-15 per plot) were removed using a soil auger to generate a 100 g sample. Samples were collected in a stomacher bag, mixed with sterile water 1:1 (100 ml water), stomached for 5 minutes, and 10-fold dilutions from 10^{-1} to 10^{-5} generated. Duplicate plates (provided by the VLA) were inoculated with 0.5 ml of each dilution and spread as described below:

Neat, 10^{-1} , 10^{-2} onto Rifampicin + Cloramphenicol plates

10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} onto Rifampicin plates

Incubation was for 24 h aerobically @ 37°C

Subsequent sampling were undertaken at 1, 2, 7 and 14 days

No conjugation was detected from the experiments in March, however the second experiments in June did show that conjugation was possible under the new conditions. Results are presented in table 5. This difference may imply that temperature as well as other climatic conditions may influence the outcome of conjugation in field conditions. We may conclude that warmer temperatures in the summer season favored the transfer of the resistance determinants by conjugation.. The figure bellow shows an example of transconjugants on selective plates. The presence of the plasmid and the assessment of the PFGE fingerprint proved that these were recipients that had acquired the resistance genetic determinants (this topic will be covered in more detail under objective 4).

Table. 5 Assessment of transfer in spread farm wastes (Field experiments)

		5 hours	24 hours	48 hours	7 days	14 days
Cattle	STMD/ECR	0	0	0	1/512 ^a , 8 ^b	0
	STMD/STMR	0	1/4231 ^a , 17 ^b	1/5011 ^a , 6 ^b	1/98 ^a , 41 ^b	1/366 ^a , 6 ^b
Pig	STMD/ECR	0	0	0	1/1700 ^a , 2 ^b	0
	STMD/STMR	0	0	1/1464 ^a , 28 ^b	1/90 ^a , 527 ^b	1/560 ^a , 10b
Poultry	STMD/ECR	0	0	0	1/731 ^a , 16 ^b	0
	STMD/STMR	0	0	0	1/263 ^a , 89 ^b	0

^aNumber of transconjugants/number of recipients. ^bNumber of transconjugants/gram of pasture sample.

Transconjugants (top: *E. coli*, bottom: *S. Typhimurium*) from field-scale spread models



OBJECTIVE 03

Assessment of transfer of resistance in treated farm wastes (primary milestones 03/02, 03/03)

Model and field scale waste treatment systems will be developed by VLA and ADAS. The systems will be used to study uptake of antimicrobial resistance from a range of viable wastes and from naked DNA in waste sterilised by the treatment or by heat.

1. Studies on transfer of naked DNA.

Preliminary experiments were carried out using heat-treatment models. Aliquots from the storage experiments were autoclaved 3 times at 121°C 15 min to break bacterial cells in order to provide a source of “free” DNA. Recipient strains (*E. coli*) were used in these models to assess the potential for DNA-uptake, no transformant cells were isolated.

In order to get a clearer idea of the mechanisms involved, we have moved to investigate more artificial laboratory conditions. **This information is provided in the report provided in Annex 10.**

2. Treatment models.

Initially, models simulating stirring tanks, anaerobic or aerobic digestion, lime addition were thought to be suitable for this section of the project. However, most farms would struggle to finance treatments of this sort. Most farms only have 1 slurry store and this store is added to on a continual basis. As a consequence a more realistic approach was

followed. Storage without extra additions is the cheapest and most pragmatic way to treat pathogen-containing manures. What we have done is to assess the effectiveness of a very cheap treatment method for manures.

An ADAS tank storage model has been used to assess the influence on the length of storage on the risk of transmission of antibiotic resistance (waste treatment study) and on the survival of pathogens. Pig and cattle slurry tanks (15,000 l) have been followed periodically up to 96 days. An on-plate protocol (modified from Smalla et al 2000 Appl. Environ. Microbiol. 66, 4854-4862) was tried out with the aim of detecting transferable elements naturally present in the samples and that could contribute to conjugation in the waste model. Five ml of LB broth were inoculated with 1 colony of the *E. coli* recipient strain (Rif^R), and incubated at 28°C o/n. Aliquots of the slurry samples (cattle or pig) were shipped to the VLA labs from ADAS on days 8, 16, 32, 64 and 96 of the storage experiment). On arrival 500 ml volumes of slurry were centrifuged for 2 minutes at 500 g and supernatant was discarded, and subsequently re-centrifuged for 40 minutes at 3000 g. The pellet was resuspended in 3 ml of LB and used as the donor population. One ml of the prepared “donors” and 1 ml of the recipient culture were mixed and plated onto a yeast extract medium plate and incubated at 28°C o/n. Thereafter, growth on the plates was resuspended in 5 ml PBS, and 10-fold dilutions were prepared up to 10⁻⁸, and plated on selective plates [McConkey with rifampicin (100 mg/l)] and McConkey with rifampicin and one of the following antibiotics: streptomycin (50 mg/l), kanamycin (100 mg/l), gentamicin (20 mg/l), or tetracycline (50 mg/l) in order to estimate the frequency of transfer. Plates were incubated for 48 h at 28°C. Trans-conjugants were isolated from the pig slurry on streptomycin and tetracycline plates on day 8 and on streptomycin plates on day 16, and from cattle slurry on tetracycline plates.

Aliquots from the tanks at the different sampling times were tested with added donors and recipients using the protocols described in the laboratory storage models. In spite of the presence of “natural” transferable elements in the waste, and of the external source of donor strains added in the model, no transfer of resistance determinants to our *E. coli* recipient strains could be detected. Data on considerations for manure treatments and survival of pathogens from long-term storage experiments is presented in Annex 7. Data from this experiment was presented at the DEFRA review meeting, also a draft paper has been produced which incorporates results from the waste treatment study

OBJECTIVE 04

Assessment of the genetic basis of acquired resistance in marked organisms (primary milestones 04/01, 04/03)

CHPL Colindale will act as the centre for characterizing resistance genes acquired by the marked organisms to confirm transfer in each of the above study models.

- 1. Production of detailed protocols for resistance characterisation (HPA, Colindale): these are provided in Annexes 3 and 9.**
- 2. Genetic characterisation of resistance mechanisms in donor organisms. (HPA, Colindale): this section is presented in Annex 3.**

OBJECTIVE 05

Development of models for transfer of resistance (primary milestones 05/02)

A full report for this topic is provided as a stand-alone document.

ADDED OBJECTIVE (requested by DEFRA).

Selected samples will also be screened following anaerobic culture to assess the possible role of anaerobic bacteria as resistance gene donors.

A protocol (Annex 8) for the isolation of resistant anaerobic organisms from animal waste has been designed and evaluated on a variety of samples. The results are summarized in the same section.

RELIABILITY AND IMPLICATIONS OF THE FINDINGS

This work has been carried out using bacteriological sampling, culture and molecular techniques using appropriate process controls and QA procedures, so the findings are robust. The main limitation of the work is having to work with a limited panel of defined organisms when in the real world there may be millions of different gene transfer opportunities. The main implications of the findings of this study is that stored animal waste does not seem to be a good environment for transfer of resistance genes by conjugation, at least this phenomenon did not occur at detectable levels. Therefore the risk can be assumed to be very small. A different situation could be presented for spread waste. Because the risk of transfer seems to be mostly dependent on the viability of the organisms when they reach the soil, it is essential to recommend that animal waste should be stored for an appropriate length of time before it is disposed onto land. This project has enabled the construction of a robust risk model where parameters can be altered and tested for future use in similar scenarios.

PROJECT OUTPUTS

2 Customer Reports, 6 presentations in national and international conferences, 1 peer reviewed paper published and 4 more in preparation. Details are provided in Annex 12

Recommendations

Annex 11 provides a list of recommendations to advise **on Reduction in Antimicrobial Resistant Organisms and Transfer of Resistance Genes in faecal waste from livestock farms.**