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Tackling AMR in the Real World- A Cross Council Initiative Theme 3:

Understanding the Real World Interactions

A Summary Report

This report has been written by Dr Jon Tyrell and Professor Matthew Avison on behalf of the Tackling AMR in the Real World programme. The opinions expressed in this publication are those of the authors. They do not purport to reflect the opinions or views of UKRI.

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Glossary

Antimicrobial Resistance (AMR)	Micro-organisms survive/remain unharmed during exposure to antimicrobial compounds- for bacteria, 'resistance' is defined using a very specific laboratory assay (a "susceptibility test") based on the ability (or not) of a specific concentration of antimicrobial to prevent growth, referred to as the "breakpoint" concentration. Breakpoints are defined for a specific bacterial species based upon, among other things, experience of successful treatment (or not) of human infections caused by that species. In some cases, breakpoints have also been defined for infections in animal species, but generally human breakpoints are used. Sometimes people wrongly refer to "resistance" when they simply mean a situation where the MIC of an antimicrobial against one bacterium is higher than against another bacterium. *The opposite to this "Antimicrobial Susceptiblity".*
AmpC ß-lactamase	Enzymes produced by Gram-negative bacteria (e.g. *Escherichia coli*) that give resistance to the critically important 3rd (though not normally 4th) generation cephalosporins. AmpC genes are found in all *E. coli* but normally the enzyme is produced at very low levels; hyperproduction (caused by mutation) is needed to give resistance. AmpC enzymes are not inhibited by clavulanic acid, and so AmpC-hyperproducing *E. coli* are resistant to amoxicillin/clavulanic acid.
Bacterial Strain	A loose term that traditionally defines multiple bacteria having the same biological properties, but increasingly refers to bacteria having close genetic relatedness, also known as bacterial lineage.
Bloodstream Infections (BSI)	Infection where viable micro-organisms are present in the bloodstream, indicated by positive culture.
CTX-M	A particularly prevalent, large and diverse subgroup of the wider ESBL class of B-lactamases
Determinant	The genetic information necessary to give an organism a particular biological function. It can be one gene, or the combination of multiple genes, and it often means specific

	variants of genes including variants that are expressed more or less than others.
Enantiomer	Two chemical variants that are mirror images of each other.
Enterobacterales	A family of bacterial species commonly found in the intestines of human and animals, and with the potential to cause infections in both. Includes E. coli, one of the most important human bacterial pathogens in the UK.
Environmental Agency	Non-departmental public body (supported by DEFRA) responsible for the protection and maintenance of the environment.
European Committee on Antimicrobial Susceptibility Testing (EUCAST)	Committee that sets the standards for the technical aspects of antimicrobial susceptibility testing that defines the breakpoint concentrations that define AMR.
Extended Spectrum ß-lactamase (ESBL)	Enzymes produced by Gram-negative bacteria (e.g. *E. coli*) which provide resistance against ß-lactam antibiotics, including the critically important 3rd and 4th generation cephalosporins. ESBL genes are typically carried on plasmids and are transferable between bacteria. These enzymes can be inhibited by clavulanic acid, and so ESBL-producing bacteria can be susceptible to amoxicillin/clavulanic acid.
Incompatibility Group F (IncF)	Incompatibility groups refer to distinct plasmid families. Two plasmids from the same family cannot reside in the same bacterium at the same time- they are said to be "incompatible". IncF plasmids are a plasmid group particularly associated with the movement of AMR (particularly CTX-M) and virulence genes.
Metagenome	The use of DNA sequencing to identify the species present (and their relative abundance) in a mixed microbial community (e.g. in a faecal sample) and in its advanced form, to identify (and relatively quantify) genes, including AMR genes, plasmids, and other mobile genetic elements (MGEs) present in that community.
Minimum Inhibitory Concentration (MIC)	Lowest concentration of an antimicrobial required to inhibit detectable growth of a bacterial population. Only when determined under specific assay conditions, and

	only if there is a relevant breakpoint defined, can the MIC be used to categorise a bacterium as AMR.
Minimal Selection Concentration (MSC)	Lowest concentration of an antimicrobial that confers a competitive advantage to a resistant-bacteria (normally defined by growth rate). Conditions for assays aiming to define the MSC are not yet formally standardised.
Mobile Genetic Elements (MGE)	Defined sequences of DNA that drive the movement of associated genes, very often genes encoding AMR. Of most relevant here: transposable elements can move genes onto plasmids; plasmids can move between bacteria.
Multi-drug resistance (MDR)	When a bacterium is resistant to at least one member from three different antimicrobial classes (though this term is often miss-used and other definitions have been proposed).
Potentially Toxic Elements (PTE)	Referring to ubiquitous trace elements within the environments (e.g. heavy metal residues) that may become toxic to other living organisms in the ecosystem at elevated concentrations.
Predicted 'no effect' concentrations (PNECs)	The environmental concentration at which a chemical compound has no antagonistic or adverse effects
Third Generation Cephalosporins (3GC)	Broad spectrum critically important (as defined by WHO) members of the ß-lactam antibiotics used to treat humans and animals, though generally reserved in humans for the treatment of serious infections. Examples include cefotaxime (human use), ceftiofur (farm animal use) and cefovecin (companion animal use), though resistance to one generally brings resistance to all.
Urinary Tract infections (UTI)	Infection within the bladder, urethra, or kidneys.
Wastewater Treatment Works (WwTWs)	Central location of sanitation and water infrastructure. Aim to clean and purify wastewater before completing water reclamation (rivers, lakes, farmland etc).
Water Framework Directive	Aims to protect and improve the quality of the water environment in the UK, and more widely, Europe.
Zoonotic transmission	The transmission of (in this case bacteria) from animals to humans either following direct interaction between humans and animals, animal faeces, faecally contaminated animal products and environments.

1. Executive Summary

Antimicrobial resistance (AMR) is now a major global public health crisis, limiting choices of viable therapeutics to treat infected patients, and as such increasing morbidity and mortality. The 'AMR in the Real World' programme is a cross-council funding initiative. The stated aim of the initiative was to provide a 'greater understanding of the role of the outdoor environment and host microbiome (entire microbial community) in influencing evolution, acquisition and spread of AMR, and as a source or reservoir of resistance'. The combined aims of the funded projects might be broadly summarised as 'investigating what factors drive the emergence of AMR bacteria within, and their spread between humans, animals and the environment'.

The data collected within the programme represent a varied picture, subject to different interpretations. This illustrates the complexity of the issues discussed and the large amount of research still needed to strengthen our understanding. For example, projects identified that current and historical antimicrobial use has a variable effect on the frequency of AMR pathogens found, their specific genetic characteristics and on the environmental influx of antimicrobial contaminants into soil and water environments. General rules of cause and effect cannot yet be defined. Whilst there is evidence for spread of AMR-pathogens between livestock animals within and between farms, subsequent zoonotic transmission between animals and humans is seemingly limited. Therefore, further work is needed to rigorously evaluate the medical significance of agricultural AMR spread. Improvements and new developments in genetic analysis that have arisen from this programme provide potential applications in these future studies.

What is clear from the work supported by this funding call is that the AMR networks of spread, and the factors driving their dynamics, are complex, varied and niche specific. This programme provides a foundation for the development of a standardised framework to understand and map these interactions, and design suitable interventions, with the caveat that this framework be accompanied with bespoke and tailored intervention strategies, reflecting the distinct parameters of the niche in question. The high-level outcomes of the programme, and remaining uncertainties are summarised as follows:

- Broadly there is variable genetic overlap for different strains of AMR clinical and livestock *E. coli*. This suggests certain strains may carry a greater risk of zoonotic

transmission and subsequent human infection. There is some evidence of strains transmitted from animals causing urinary tract or bloodstream infections (UTI/BSI) during the period of study, but this was rarely found to occur. Impact of animals on bacterial colonisation and the potential to influence resistant infections in the future remains under-evaluated and may require long-term, high resolution surveillance programmes to resolve.

- There is clear evidence of farm-to-farm transmission of bacterial strains and antibiotic-resistance genes amongst major livestock species, including cattle and pigs. Further investigation is needed to detail the extent of this spread, and to identify the contributing factors to this.
- High similarity between bacterial communities within a single livestock farm was identified. However, there was greater diversity between bacterial communities when comparing different farms, highlighting the localised nature of AMR spread in livestock agriculture.
- Genetic carriers of AMR genes are highly diverse, independent of the localised environment being sampled. The contribution of plasmids and other mobile genetic elements (MGEs) to AMR gene transmission between sampled environments remains to be explained. The potential role of plasmids and other MGEs that are not currently directly involved in AMR spread is an important area of future research.
- Trace concentrations of antimicrobials in the environment are highly selective for AMR determinants and drive their persistence in the environment.
- Environmental factors such as temperature have variable direct influence on the selection of AMR bacteria but do influence the abundance of AMR bacteria. For example, higher environmental temperatures were associated with higher abundance of resistance in some studies. It remains to be determined how widespread this phenomenon is.
- There is strong correlation between potentially toxic elements (PTEs) such as heavy metal ions and the presence and abundance of certain AMR genes and MGEs in urban areas and associated rivers. This indicates that human pollution may have a role in driving environmental selection of AMR. Further investigation into the role of PTEs is needed.

- Many of the findings highlight the relative importance of local vs regional vs national vs global strategies to combat AMR. What are the best adaptive and integrated hierarchical approaches to control that can be adopted? For example, in livestock agriculture, local, bespoke and targeted biosecurity strategies will seemingly be most effective in elucidating niche-specific transmission/selection dynamics. Local molecular surveillance of AMR may allow bespoke antibiotic usage strategies at farm level to reduce AMR in a targeted manner. However, the features of AMR in a niche may change rapidly over time, and as such flexible approaches to intervention would be desirable.
- A number of projects have optimised technological approaches to analysing AMR and developed novel strategies which would benefit from *in vitro* and *in situ* replication. Such innovations possess much promise to be developed further in future action plans.
- It is clear that increased intervention-planning, farmer/stakeholder involvement and relating this work to clinical/environmental scenarios will improve the impact of the programme's findings. This could include efforts to gain more detailed antibiotic-use histories in local farming communities, so as to add real context and relevance to datasets collected on the potential selection pressures of AMR in these backgrounds.

2. Introduction

2.1 Antimicrobial Resistance (AMR) as an archetypal One Health Issue

Historically, antimicrobial resistance (AMR) in the UK was seen as a predominantly human healthcare associated issue: antibiotics are used to treat infections in humans, bacteria residing within humans become resistant to treatment, a new antibiotic is selected, and repeat. It wasn't until the 1970s that the idea that antibiotic use in farming might drive the emergence of resistant bacteria that, as well as affecting farm animal welfare and production efficiency might potentially pose a zoonotic threat to humans. However, the global expansion of AMR mechanisms such as the extended-spectrum β-lactamases (ESBLs), and particularly CTX-M enzymes, which are encoded on mobile genetic elements and can move between bacteria as well as be carried by a particular bacterial host, revealed the importance of community, zoonotic, and environmental multi-directional and international transmission

pathways (Seiffert *et al.*, 2013; Pitout *et al.*, 2005). As such, the 'One Health' research framework has been employed to help define, understand and manage the scope and complexity of the AMR transmission network across human, animal and environmental sectors.

The One Health Commission (OHC) defines 'One Health' as a 'collaborative effort of multiple health science professions, together with their related disciplines and institutions - working locally, nationally and globally - to attain optimal health for people, domestic animals, wildlife, plants and our environment'. AMR in bacteria is an archetypal One Health research problem, with considerable global impacts on human and animal health, food security and safety, and our ability to meet strategic development goals. Increasingly, evidence suggests AMR is an issue that may be further exacerbated by climate change. The One Health framing of AMR research deals with the dynamics of bacterial populations and mobile AMR genes between different "compartments" (e.g. a pig farm, a hospital, a river) within a complex transmission network. The emergence, persistence and dissemination of AMR may be variably influenced by different biological, chemical and environmental parameters existing in each compartment and by how the compartments are physically connected. Figure 1 illustrates some of the transmission pathways interlinking generalised compartments and known major drivers of AMR.

Much progress in international One Health initiatives has been made in recent years, including those undertaken by the European Commission (https://tinyurl.com/nrnhu64b) and the World Health Organisation (WHO) (https://tinyurl.com/573ad7c9). Furthermore, the work described herein summarises new approaches and insights gained in relation to AMR in the real world within the remit of One Health, enabled by the cross-council funding initiative targeting this important problem. Despite these advances, the hugely complex nature of the problem of AMR mean that many gaps in our knowledge remain, some of which are highlighted below.

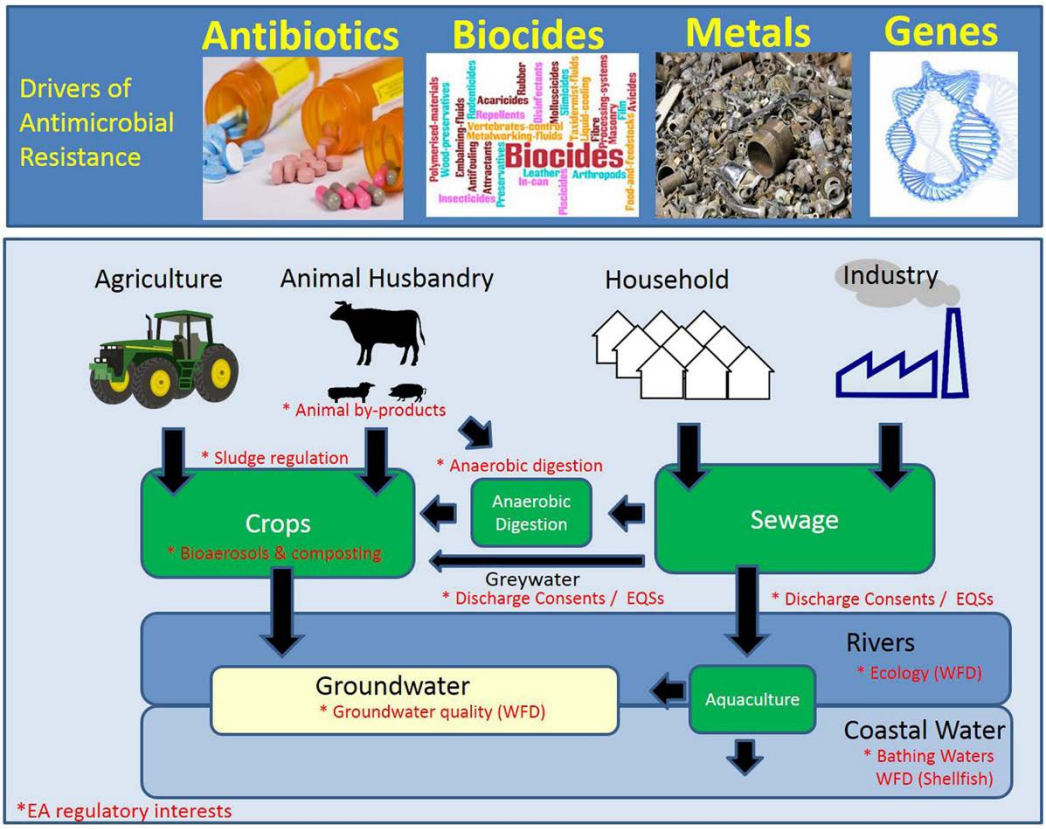

Figure 1*: Representation of potential routes and dynamics within the One Health AMR Transmission Network* (Singer *et al*., 2016). EA: Environmental Agency, WFD; Water Framework Directive, EQS; Environmental Quality Standards.

2.2 Introduction to the Programme and Aims of the Funding Call

This programme was part of the UKRI's AMR cross-council initiative, co-funded by NERC, BBSRC and MRC. The over-arching aim of the programme was to achieve 'greater understanding of the role of the outdoor environment and host microbiome in influencing the evolution, acquisition and spread of AMR, and as a reservoir for resistance'. This aim was partitioned into two work packages;

- WP1: understanding the acquisition, spread and evolution of AMR in the environment, and how this affects exposure risks for humans and animals
- WP2: understanding of the acquisition, spread and evolution of AMR in the host microbiome (human/animal).

A total of 4 consortium research projects and 9 pump-priming projects were funded through this call and made up the programme integration group that developed this report. Details of those contributing to the report can be found in Table 1.

2.3 Aim of the Report

The report summarises the key research findings, and future implications of the projects outlined above, in the context of the original aims and objectives of the funding call. Discussions will be framed around key narratives and insights that have emerged from the programme relevant to environmental management, livestock agriculture practice, the involvement of stakeholders to implement impact and innovations in our research strategies and capabilities that will provide insightful opportunities moving forward. We also consider key remaining knowledge gaps, supporting prioritisation of future AMR funding calls. This is a vast and complex research space which will require focused and concerted efforts from multi-disciplinary research teams for decades to come before we can fully understand enough to accurately predict and broadly mitigate the future impact of AMR. Additionally, because the AMR landscape is inherently evolving and highly plastic, approaches to surveillance and interventions need to be sufficiently flexible to adapt.

Table 1: *Contributing Projects*

	Project Code	Project Lead	Project Title
Consortia Research Projects	**[P1]**	Prof. Dov Stekel [dov.stekel@nottingham.ac.uk]	EVAL-FARMS: Evaluating the Threat of Antimicrobial Resistance in Agricultural Manures and Slurries
	[P2]	Dr Andrew C. Singer [acsi@ceh.ac.uk]	Chicken or the Egg: Is AMR in the Environment Driven by Dissemination of Antibiotics or Antibiotic Resistance Genes
	[P3]	Prof. Matthew Avison [matthewb.avison@bristol.ac.uk]	Acquisition and Selection of Antibiotic Resistance in Companion and Farmed Animals and Implications for Transmission to Humans
	[P4]	Dr Nicole Stoesser [Nicole.stoesser@ndm.ox.ac.uk]	The environmental REsistome: confluence of Human and Animal Biota in antibiotic resistance spread (REHAB)
Pump-priming Projects	**[P5]**	Prof. Charles Keevil [c.e.krrvil@soton.ac.uk]	Occurrence and horizontal gene transfer of carbapenemase and ESBL genes in soil microbiomes
	[P6]	Dr Charles Knapp [Charles.knapp@strath.ac.uk]	Quantifying Spatial AMR Patterns across Urban and Rural Landscapes
	[P7]	Dr Emily Rousham [E.K.Rousham@lboro.ac.uk]	Spatial and temporal dynamics of AMR transmission from the outdoor environment to humans in urban and rural Bangladesh
	[P8]	Dr Igor Morozov [ab6069@coventry.ac.uk]	Identification of novel double-stranded RNA elements in developing antibiotic resistance in the agricultural environment
	[P9]	Prof. Barbara Kasprzyk-Hordern [b.kasprzyk-hordern@bath.ac.uk]	Impact of stereochemistry of antimicrobial agents on their environmental fate, biological potency and the emergence of resistance
	[P10]	Dr. Alex Corbishley [alexander.corbishley@roslin.ed.ac.uk]	The dynamics of antimicrobial resistance gene prevalence on a commercial pig farm: implications of policy
	[P11]	Dr Jennifer Ritchie [j.ritchie@surrey.ac.uk]	A quantitative method to evaluate AMR distribution in complex communities based on methylome profiling
	[P12]	Prof. Fiona Henriquez [Fiona.henriquez@uws.ac.uk]	Genes of past, present & future: does legacy pollution contribute to antibiotic resistance in industrialised estuaries?

3. Implications for Livestock Agricultural Practice

Slurry storage represents a potential way for large dairy farmers to reduce levels of AMR bacteria entering the environment

Many species of bacteria that are important potential human and animal pathogens are caried in the intestines of animals and so are regularly excreted. Slurry (a mixture of faeces, urine, water and other farm waste) management is a key process within the livestock farming environment, recycling livestock waste into reusable fertilising material in order to replenish and promote crop growth and grazing fields. However, slurry also represents a potentially important faecal-to-environment transmission route for potential zoonotic pathogens, including those carrying AMR determinants. In [P1] the composition of dairy farm slurry collection and storage tanks in regards to presence and persistence of bacterial communities and AMR determinants were analysed. Using *Escherichia coli* as an exemplar organism, alongside detailed metagenomic analysis, it was shown that microbial communities and AMR gene content are stable in a given slurry tank, if in dynamic but stable equilibrium driven by the balance of microbial death within the slurry tank environment, and the addition of fresh organic material from cattle on the farm. The AMR gene content of slurry in some way reflects historical antibiotic use on farms, suggesting that storage of slurry, without fresh input, has an amelioratory role, with resistance generated/selected for *in vivo*. Modelling analysis suggested that the impacts on AMR prevalence of reducing total antibiotic usage on a farm below what is currently seen as a responsible level may vary. Whilst high-antibiotic use farms may gain benefit from significant reduction in antibiotic usage, low-antibiotic use farms may gain more significant benefits through alternative more localised strategies not related to antibiotic use (e.g. reducing the transmission of AMR bacteria within the farm).

In [P1] it was also shown that careful slurry management, through a two-tank, 90-day storage approach, predicted a significant reduction in the rates of AMR bacteria present, and the numbers of *E. coli* resistant to antimicrobials such as cefalexin or amoxicillin which are commonly used for the treatment of infections in farmed animals, companion animals and humans. If replicated, this represents a potential management intervention, where logistically viable at least, that could be applied to reduce AMR in cattle farms nationally, in particular for larger dairy farms which are capable of capitalising changes in practice. In an industry facing increasing consolidation, these findings will become relevant to an increasing

proportion of farms. A notable limitation is that this work was carried out upon a typical high-performance university farm with controlled environments, where rates of animal disease and antibiotic usage are low. Whilst metagenomics were used to reflect the diversity and range of bacterial species present, further such studies on less stringently controlled farms may further confirm generalisability of these findings.

Seasonal effects on AMR selection and transmission vary based on multiple additional parameters

Project [P3] looked specifically at the presence of AMR-*E. coli* within faecal samples of animals of different ages in dairy farms. A primary finding was that AMR was most abundant in calf faeces. This was, however, reduced in farms with more regular washing out of water troughs (i.e. once a day), suggestive of another management practice that may reduce on-farm AMR prevalence. AMR in faecal *E. coli* was also affected by environmental temperature; average monthly temperatures in February and September recorded the lowest and highest AMR rates respectively, including resistance to numerous antibiotic classes including the critically important (in human medicine) fluoroquinolones and 3rd generation cephalosporins. Also, in [P3], outside pasturelands were shown to be less likely to be contaminated by AMR E. coli (as a proportion of total E. coli) than indoor hosing. This may also be due to a temperature effect: it is generally warmer inside. The explanation for why temperature influences AMR prevalence requires experimentation to define, but it could be due to temperature-driven difference in the fitness costs imposed on bacteria by the expression of AMR, or in AMR plasmid transmission dynamics. One implication of this finding is that global warming will increase the burden of AMR in natural and farmed environments.

In [P4], seasonal variation of temperature was shown to have limited effect on the bacterial community makeup of samples collected across a wide range of farm and environmental sites, with persistent strains reported across all timepoints. However, seasonal fluctuations in human and poultry colonisation with ESBL-positive (3rd generation cephalosporin resistant) *E. coli* in Bangladesh were statistically significant with higher prevalence in the wet monsoon season, compared to the cooler, dry months of winter [P7]. Both species/strain variation and size of microbial communities are important factors when interrogating AMR transmission at both a population and genetic level. Evidently further

modelling and rigorous investigation of these particular dynamics, and implications for farm management, is warranted.

Antimicrobial use selects for AMR bacteria on farms, and reduction of use to zero is unlikely to eliminate AMR alone

Overall, [P3] found that across 53 dairy farms under study, when taking into consideration variations in farm management practice using sophisticated statistical analysis, antimicrobial use had only a weak impact on relative levels of AMR-*E. coli* found, with only a small number of specific effects being observed. Whilst higher (critically important) fluoroquinolone antibacterial use correlated with higher prevalence of fluoroquinolone resistant E. col , this was only observed in calves and not in adults. Increased use of amoxicillin-clavulanate was associated with increased rates of *E. coli*, hyperproducing the β-lactamase enzyme AmpC, which are amoxicillin-clavulanic acid resistant but also resistant to (critically important) 3rd-generation cephalosporins. Hence moves to reduce the use of critically important cephalosporins, resulting in the switch to amoxicillin-clavulanic acid use instead, may result paradoxically in the increased abundance of 3rd generation cephalosporin resistance. The abundance of *E. coli* producing CTX-M β-lactamases (providing 3rd and 4th generation cephalosporing resistance) was associated with increased use of 4th generation cephalosporin dry cow therapies, but again only in calves. The alternative use of a non-critical aminoglycoside (framycetin) as a dry cow therapy was also associated with increased CTX-M rates in calves through co-selection of particular AMR plasmids carrying CTX-M and framycetin. Across 14 farms (encompassing 3 major livestock species- pigs, cattle, sheep) evaluated with greater within-farm sampling resolution, [P4] identified significant associations between total and class-specific antimicrobial use and resistance to aminoglycosides, ß-lactams, phenicols, sulphonamides, tetracycline and trimethoprim. Additionally, there was a risk of multi-drug resistance (MDR) associated with increases in the number of different classes of antimicrobials used on-farm. Livestock-specific associations were also observed, with pig-farming associated with the highest risk of MDR isolates on-farm, and sheep-farming the lowest. [P10] looked more closely at pig farming, specifically comparing sow and young piglet sampling. Results showed a high abundance and diversity of AMR genes. However, in-feed antimicrobial dosing seemingly had little impact, suggesting a

stabilisation due to historical antimicrobial use. Clearly, there is complicated and potentially sector-specific influence at play that needs further elucidation.

Small-scale commercial poultry farms in Bangladesh had high and unregulated antibiotic use, sometimes including colistin, throughout the production life cycle [P7]. *E. coli* isolates from such poultry across 20 farms were variably found resistant to fluorquinolones (88%), sulphonamides (80%), aminoglycosides (42%) and carbapenems (11%). Many of these are on the WHO critical list of antibiotics for human use. The resistance profiles were significantly different in farmed poultry samples to those in domestic, free range poultry from local households with no history of antibiotic use.

Secondary effects of antimicrobial treatment were further highlighted in [P8]. Antimicrobial therapy was correlated with a number of significant livestock co-morbidity indicators and to expansion of *E. coli* colonisation in the test calves.

Whilst much evidence supports the recently introduced Red Tractor critically-important antimicrobial use regulations (https://assurance.redtractor.org.uk/standards/search?c=9), deeper investigations suggest broadscale policy choices may have potentially unforeseen consequences, akin to the example of paradoxical co-selection of critically important resistance due to switching to certain non-critically important replacements, as described above. Furthermore, a working hypothesis from the results of [P3] is that antibiotic dry cow therapy contaminating colostrum, which inadvertently provides am antimicrobial dose to new-born calves, potentially increases selection for certain AMR bacteria, whilst reducing the prevalence of others. This could prove critical to the long-term presence of resistance on the farm since dairy heifer calves are usually retained on the farm as replacement milking cows. Further research under controlled conditions is required to confirm this hypothesis, but it may lead to interventions designed to match dry cow therapy use to the ecology of AMR bacteria on the farm, thereby driving down the prevalence of critically important AMR in the medium term.

Transmission routes within farms, and between farming locations, are evident

Preclusion of over-generalised management strategies, and evidence in support of localised interventions was suggested in the outcomes of [P4]. [P4] used an in-depth pangenomic

analysis, encompassing the entire genetic content within the samples, across several Enterobacterales species. In doing so, it illustrated that there was significant distinct diversity in AMR genes and plasmid contexts across One Health compartments included in their project, with some evidence of transmission between these compartments. In an analysis of the IncF plasmid family, commonly associated with AMR genes, and particularly CTX-M genes, rapid flux of non-core plasmid genes was likely key to enabling rapid niche adaptation, contributing to successful dissemination across niches. Though individual niches (e.g. livestock, species, farms) maintained distinct population structures, these could facilitate the emergence of different AMR mechanisms in response to localised selective pressures. These could then be disseminated through inter-niche transfer of bacterial strains. This risk of dissemination was also reported in [P8] which highlighted a case of the transmission of a colistin-resistant determinant *mcr-2*, which had been vertically transmitted from mother to calf, before international trading of the calf.

[P4] is currently investigating the extent of genetic overlap of WwTW and livestock Enterobacteriales and AMR determinants with those found in human bloodstream infections (BSIs) in the same region. Notable, BSIs represent the 'tip-of-the-iceberg' of any human gastrointestinal colonisation event, with clearly many more colonisation events occurring than those that manifest as invasive infections. Preliminary data suggest clear overlap across compartments and occasionally across Enterobacterales species for certain mobile genetic element (MGE) sub-populations, especially plasmids and smaller MGEs. Similarly, this is observed for some bacterial lineages (e.g. *E. coli* ST10), with intermingling of isolates within phylogenies, albeit with relatively large genetic distances between individual isolates from animal/environment vs human niches. In investigating human urinary *E. coli*, [P3] found little evidence of zoonotic transmission of epidemiologically significant AMR bacteria or genetic determinants directly resulting in UTIs. UTIs often result from established colonising flora; as such it is difficult to ascertain the impact of current zoonotic transmission and human colonisation on the development of future resistant infections in humans. Nonetheless, genetic data available in [P3] suggest that transmission of critically important of 3rd generation and/or fluoroquinolone resistant *E. coli* strains implicated in UTIs predominantly arise from person-to-person interactions. The possibility of variation by setting was highlighted by work undertaken in areas of low biosecurity in Bangladesh, [P7], where high

rates of colonisation with 3rd generation cephalosporin resistant, ESBL-positive *E. coli* across human, environmental and animal (poultry) compartments were observed. However, no significant difference was seen in human colonisation rates between cohorts with high/low exposure to poultry.

4. Implications from Environmental Management

WwTW represent source of environmental AMR indicators

Wastewater from the human populace is a major route of clinical antimicrobial chemical pollution into the environment (Rizzo *et al*., 2013). [P9] surveyed the prevalence of fluoroquinolones (and an associated resistance gene *qnrS*) in treated water from 5 different WwTWs serving the catchment of 1.5 million people. Fluoroquinolones were found ubiquitously within water samples and frequently at high quantities (often >100g/day), as was *qnrS*. There was disparity in the degree that fluoroquinolones (particularly in the case of ciprofloxacin and ofloxacin) and *qnrS* were removed by difference wastewater treatment procedures. Where trickling filter technology was used, removal of fluroquinolones was poor, but a significant reduction in *qnrS* genes was observed. The opposite was true for WwTWs using 'activated sludge' procedures (Castrignano *et al*., 2020). [P9] undertook a wider monitoring campaign focussed on the abundance and distribution of 16 antimicrobials (including the critically important fluoroquinolones and macrolides) and 4 corresponding AMR genes sampled from the influent to five WwTWs within a single river catchment. Most antibiotics were detected within all influent wastewater from the 5 cities, as were the corresponding AMR genes. Strong positive correlations were observed between the daily loads of antibiotics and AMR genes versus the size of the population served by each WwTWs, as well as the between antibiotic and AMR gene loads at a single site. The efficiency of antibiotic/AMR gene removal by WwTWs varied according to site (and treatment process utilised) and target. Strong correlations were maintained between the population size served by the WwTWs and daily loads of discharged antibiotics/AMR genes into the environment. The results indicate that population size is the main determinant of the magnitude of the antibiotic and AMR gene burden in the environment.

At a broader level, analysis of influent and effluent metagenomics at 5 WwTWs and a downstream gradient from these in [P4] consistently demonstrated that influent samples had

the highest richness and abundance of AMR gene variants, insertion sequences and Enterobacterales plasmids than other sample types along this gradient, suggesting that WwTW reduce, but do not prevent, environmental contamination. Variable impacts were identified for different AMR genes and MGEs. It is clear that developing a better understanding of the impact of different wastewater treatments procedures on the reduction of relevant bacterial, AMR genes and chemical compounds in municipal waste is crucial.

Environmental trace concentrations of antibacterial compounds carry a selective risk for AMR determinants

Wastewater management in addition to leaching from livestock agriculture, clearly plays a role in environmental contamination with AMR bacteria, AMR genes, and antimicrobial chemicals of clinical and veterinary origin. What is less understood is the significance of this in the dissemination and persistence of AMR within the environment. [P2] addressed these questions by artificial, *in situ* and *ex situ* riverine simulations, focusing on the impact of fluoroquinolone and macrolide antibacterial contaminants. Fluoroquinolones (such as ciprofloxacin) are amongst the most widely prescribed antimicrobials for humans, and also listed on the European Commission's Water Framework Directive's (WFD) priority hazardous substances watchlist. In short, [P2] illustrated that measured environmental concentrations (MECs) of selected antimicrobials are sufficient to select for resistance to these antimicrobials in both clean and polluted scenarios. Using experimental conditions within wastewater influent, the impact of trace-level ciprofloxacin was observed through significant taxonomic changes in microbial communities, and increased prevalence of MDR efflux pump genes implicated in the generation of MDR phenotypes within these bacterial communities. A minimal selection concentration (MSCs) of 0.003ug/ml of ciprofloxacin was recorded, much lower than the clinical breakpoint concentration (>0.5ug/ml) that defined ciprofloxacin resistance in *E. coli* (defined by the European Committee on Antimicrobial Susceptibility Testing, EUCAST). Comparative MSCs were recorded across both simulated and natural riverine experiments, consistent with the fact that; (i) MSCs of antimicrobials may not be as dependent on environmental, temperature and nutrient parameters as previously thought, (ii) there can be confidence in the 'real world relevance' of experimental data sets for these antimicrobials, providing the opportunity for more rigorous investigation of risk assessment

and community modelling. Interestingly, whilst MSCs were of the same magnitude across the experimental set ups, the effects on AMR prevalence and microbial community structures conferred by exposure to the MSC varied by setting. Microbial responses in laboratory set-ups conferred increases in particular sets of AMR genes, whilst river experiments inferred predominantly phenotypic responses in MDR efflux systems. It was also evident in [P2] that different AMR genetic determinants respond differently to the same selective pressures. Further work to more accurately model the complex communities and fluctuating environmental parameters would be required to interrogate these differences, and their wider implications.

Wider experimentation under a more varied set of parameters, and a wider spectrum of antimicrobial selectors and degradative intermediaries, including mixtures, would contribute a deeper understanding of the environmental selection of AMR organisms and determinants at the microbiome level. [P9] deals with the occurrence and bio-physiochemical transformation of selected fluoroquinolones and their metabolites in wastewater and receiving waters as well as a wider group of antibiotics in a large scale study focussed on the Avon catchment and 5 local WwTWs that represent >75% of the wastewater from the catchment population. Positive significant correlations were observed between the combined daily loads of all antibiotic classes measured, daily number of AMR gene and population size served by each individual WwTWs. This again indicates population as a key driving factor on levels of antibiotics and AMR genes in influent wastewater. The removal of antibiotics and AMR genes during wastewater treatment was found to be site and target specific, and highly variable. It is therefore difficult to draw conclusions regarding possible impact on AMR. However, it is important to note that selection for particular genes (sul1, qnrS) does occur. Further work is required to understand the role WwTWs play in the dissemination of AMR.

Most antibiotics are chiral. Whilst the impact of stereochemical variants/enantiomers of antimicrobials and their interaction with biological systems is carefully monitored in the pharmaceutical sector, there is a paucity of such understanding within the environment. Enantiomers are simply different configurational variants of the same compound, which might result in different biological potency of different variants as well as their impact on AMR. [P9] and previous work (Castrignano *et al*., 2018) evidences the presence of enantiomeric ofloxacin (a fluoroquinolone) in wastewater effluent. Enantiomeric profiling

revealed that ofloxacin was enriched one particular chemical variant, likely derived from its prescription as the more potent version of pure levofloxacin, alongside racemic ofloxacin. While ofloxacin's enantiomeric fraction (EF) remained constant, high stereoselectivity was observed in the case of its metabolite ofloxacin N-oxide. In short, this indicates that two enantiomers of the same antibiotic are subject to different biological processes which might be of significant consequence regarding AMR but further work is required to determine any role these stereochemical variations may play in dynamic AMR selection and transmission processes i.e. do different stereochemical variants of an antimicrobial have different effects on the selection of AMR, and the subsequent transmission. [P9] also investigated the antibacterial chloramphenicol as a proof-of-concept to demonstrate the importance of stereoselective metabolism by environmental AMR bacteria. It was shown that chloramphenicol was subject to extensive chemical transformation due to metabolism of environmental bacteria (Elder *et al*., 2021)

As described above, [P2] identified that environmental variations (temperature etc) seemingly had minimal effect on the MSC of the fluoroquinolone ciprofloxacin, albeit within the specific riverine niches of the study. Preliminary metagenomic analysis from [P4] suggests in contrast that seasonal effects on the taxonomic composition of influent, effluent and river sediment may be observed. In studying the prevalence of AMR bacteria in cattle, [P3] found a statistically significant negative correlation between prevalence of *E. coli* resistant to five different antimicrobials tested, as *E. coli* carrying the CTX-M-positive faecal samples, and cold seasonal temperatures. This has a number of implications. Firstly, that point-prevalence studies risk unconsciously biasing results depending on what month sampling is undertaken- longitudinal studies are warranted. Secondly, on a wider scale, this hints at the growing relevance of increasing temperatures and climate change to the rapid expansion of AMR pathogens.

In particular, the work of [P2] and [P9] allow more informed environmental modelling and risk assessment regarding the threat posed by antimicrobial residues. Several antibiotics were found exceeding environmental PNEC's in wastewater influent and effluent, though to a lesser extent in receiving waters. This effect was found to differ depending on the antimicrobial in question, thus investigation is required to fully understand the risks of particular antimicrobials within specific catchment niches. Further work has progressed from

[P2], using improved water tank models, with wider experimental parameters, at the UKCEH (UK Centre for Ecology & Hydrology). What though, if any, are the tangible effects environmental contamination of trace antimicrobials may have? [P4] illustrates that in samples collected in WwTWs areas, there was a far higher diversity of *E. coli* phylogroups and AMR plasmids than in livestock samples. This may however be attributable to the combined input to wastewater sites from human, livestock and environmental sources, when compared to livestock niches. [P4] found no notable overlap between *E. coli* populations of livestock and (potentially contaminated) WwTWs, suggesting that this is not a primary transmission route of AMR to or from farm environments. It is important to note, however, that culture-based approaches capture only a fraction of the whole diversity present in any of these polymicrobial samples, and supplementary insights may be gained from accessing and evaluating deep metagenomic datasets.

Environmental contaminants carry a co-selective threat for AMR

The co-localisation of metal-resistance genes and AMR genes on the same MGE e.g. plasmids, has long led to heavy metals being implicated in the co-selection of AMR-bacteria. Looking at total and bioavailable levels of potentially toxic elements (PTEs) such as metals and metalloids, [P6, P12] aimed to ascertain and describe potential correlations between PTEs, and their role in the environmental selection of AMR genes and related MGEs. Results show that PTE levels were highest within urban areas, and aquatic environments perhaps unsurprising given societal and industrial pollution sources. Primarily, positive correlations between PTEs, especially bioavailable ones, and the presence of AMR genes was identified in urban soils- perhaps suggesting an undervalued source of AMR selection in environmental settings and one that should be further monitored, particularly where in close proximity to agricultural landscapes. Pollutants and AMR genes are also found in deeper sediments, suggesting a role of legacy pollution in driving AMR in the environment [P12]. Interestingly, a positive correlation of PTEs with MGEs (but not the AMR genes themselves) was recorded, perhaps indicating a secondary role in driving AMR gene transmission through amplification of MGE populations. Similar pollution-impacted patterns between PTEs (and including exposures to polycyclic aromatic compounds and persistent pollutants) were seen across Scottish landscapes in [P6]. Work on a wider scale is clearly needed to evaluate the threat

posed to agricultural AMR transmission by secondary anthropogenic pollution represented here.

5. Implications for Stakeholder Involvement

Observations on the nature of future stakeholder involvement

Given the real-life applications of the programme in regards to both policy development, wastewater management and livestock farming practice, it is increasingly important to maximise stakeholder involvement. Further effort for co-produced communicative and sampling networks may provide richer data sets, and also provide more lasting impactful interventions downstream when the findings from this programme are applied into policy and practice.

Such practices rely on accurate and mutually beneficial communication between research and farmer stakeholders, and the language used to facilitate this. For example, [P1] participants identified that clinical and wider societal implications of AMR often are not perceptible to farmers, and even, often, veterinarians. Clinical AMR is not an overly perceptible concept to dairy farmers or indeed to veterinarians, and the use of certain 'buzzwords' often represented a barrier (Helliwell *et al*., 2019; Helliwell *et al*., 2020). Buzzwords such as 'hotspots' and 'reservoir' are often used vaguely, without clear or robust meaning, and often are perhaps not the most accurate terminology for the loci they are intended to describe: slurry tanks, WwTWs, wider dairy farm environments for example.

Stakeholder involvement represents chance for improved impact and legacy

Stakeholders have been engaged in this project and REF impact case studies are under development. [P7] is unique within the programme in having a central role for formal stakeholder involvement, whilst also being international in nature. Using a multifaceted approach of in-depth interviews and structured observations, [P7] was able to examine cultural and social practices of animal husbandry, identifying key routes of potential AMR transmission between important silos across 3 sites: urban markets, commercial poultry farms and rural villages. This approach gave a deep insight into the bespoke practices and risk factors of cultural behaviours. Many areas of limited, or negligible biosecurity were identified. The findings fed into a point prevalence observational study mentioned previously. Whilst the

minutiae of farming practices in Bangladesh may not be directly relatable to those in the UK, the multi-disciplinary approach, integrating microbiology, epidemiology and social science, represents an opportunity to develop a model for future progressive studies, for more informed study design and delivery. There is an opportunity to drive findings from multiple projects within this programme into practice. For example, follow on work in the form of observational trials are currently ongoing on 20 dairy farms to investigate the impacts of antimicrobial usage and management practice interventions identified from the findings of [P3] on AMR prevalence on UK dairy farms.

6. Innovations of Study Design & Methodological Capabilities

Further innovation in methodological capabilities to adequately investigate the complex microbial communities, transmission dynamics, and external influencing factors that dictate AMR transmission between agricultural and environmental compartments is still required and reflected in several projects.

[P11] details a novel quantitative approach to evaluating complex communities based upon 'methylome profiling'. The project identified that methyltransferase labelling may be used as an active marker for plasmid carriage and transfer within control strain populations (although technical issues related to sequencing depth and read length, masked the signal in more complex, less abundant *in vivo*-derived microbial communities) or mapping the rate at which plasmids are transferred, in line with the original call. Optimisation of methylome profiling may hold potential for future applications in interrogating complex populations, and to better monitor the acquisition and loss of AMR-plasmids, the significance of which is still not well-described within a wider transmission network.

Throughout this programme, the complexity of genetic factors at play, including AMR genes, MGEs, and strain level interaction has been evident. The complexity of microbial communities within samples makes analyses challenging. [P4] carried out pilot investigations into optimising the way in which shotgun metagenomic approaches may be used to infer taxonomic composition and AMR gene content within these settings. Whilst most environmental studies similar to those outlined here, use sequencing depths of 10 million reads per sample, [P4] showed that even at depths as high as 200 million reads per sample the full diversity of AMR in a sample was not ascertained. It is clear then that further

optimisation of sequencing depth, as well as the subsequent data processing and annotation, is required. As a minimum, results for less-deep metagenomic experiments should be appropriately caveated, effectively enabling a 'rule-in' of AMR gen presence in a sample, but not of absence, at lower sequencing depths. Put simply, one benefit of this technological advance is that the increasing prevalence of AMR genes in samples collected at a certain location over time might be observed through very deep sequencing, but with less deep sequencing the AMR gene might have been missed until its prevalence crossed a threshold where it could be detected. The AMR gene might then appear to have been transmitted into the location at a particular timepoint, but in fact it was there all along and increasing in prevalence due to selection. Metagenomic analyses can complement culture-based (phenotypic) AMR screening methods, which would add further resolution and perspective. Indeed, in [P8] transcriptome analysis showed that the abundance of anti-sense RNA, a proxy to AMR gene expression, does not directly correlate to the overall genetic copy number of AMR genes present; hinting at a silent 'resistome' that may not be detected with phenotypic-based screening methods. Additionally, it was found in [P10] that the antibiotics chlortetracycline and tylosin were still clinically active in pigs, despite high abundances of corresponding resistance genes being found. This programme has given rise to a unique learning opportunity in the development of sequencing approaches and data analysis which can inform similar studies in the future.

7. Concluding Remarks

7.1 Policy Implications

In 2019, a 5-year action plan and a 20-year strategy to fight AMR were published by the UK Government (national action plan, NAP), led by DHSC (Department of Health & Social Care), PHE (Public Health England), DEFRA (Department for Environment, Food & Rural Affairs) and VMD (Veterinary Medicines Directorate) (https://www.gov.uk/government/collections/antimicrobial-resistance-amr-information-and-resources). These plans highlight and address central domestic and global areas of threat for AMR emergence and spread, and translate that into driving evolved policy, advocacy and political agenda.

These documents outlined key ambitions that are reflected in the underlying aims of this programme (Figure 2). The programme represents a detailed, interdisciplinary consortia of work that cuts across and supports a number of key national ambitions represented in the NAPs.

The work investigating antimicrobial contamination of environmental and water compartments of the transmission network, aiming to help us understand the potential selective impact of these chemicals on AMR communities, and the wider implications of such (see Ambitions 1, 3, 6). Participants in this programme are actively working with the UKCEH and regulators at both national and international level to further inform the risks posed by environmental antimicrobial residues. This programme has furthered our understanding of the role played by contemporary and historical antimicrobial use within livestock agricultural settings. This will further inform improved localised and bespoke antibiotic stewardship measures (Ambitions 7, 8) and avoid livestock infection through reduced selection of AMR pathogens (Ambitions 3. 5. 6). Datasets fundamental to contextualising and deepening our knowledge of the global AMR transmission networks in question have been collected and provides a strong foundation for further research (Ambition 1). A number of key innovations have been investigated and advanced to strengthen the rigour and impact of future programmes (Ambition 2). In short, this programme provides valuable insight into the complex and plastic dynamics of AMR selection, transmission and persistence within human, animal and environmental sectors.

Figure 2: *The UK's nine ambitions for change, as defined in the UK 2040 AMR Vision (adapted from 'Tackling antimicrobial resistance 2019-2024: The UK's five-year national action plan)*

7.2 The Future

This programme of projects provides a varied and dynamic investigation into the complex One Health network involved in promoting the emergence, persistence, selection and transmission of AMR bacteria and the genes that contribute to AMR, across environmental, agricultural, human and animal compartments and contributes much knowledge to the field. As a result of the research, additional questions may be posed to further advance this wide and complex field of investigation, as highlighted throughout the report.

Whilst many studies have undertaken steps to elucidate the complex polymicrobial communities of the environmental and farm compartments in question, in general across the programme *E. coli* has been used as the sentinel organism. Whilst this has provided significant

benefits at a programme level, a wider net must continue to be cast in terms of bacterial species diversity to truly understand the microbial and genetic dynamics at play in AMR transmission. This programme, and future studies akin to it, add valuable quantifiable data to support the development and application of more sophisticated modelling approaches to provide a more rigorous analysis and stronger predicting power of the processes and parameters that contribute to AMR emergence, persistence and spread within the relevant compartments. Examples of such can be seen with Booton *et al.,* (2021) and Niewiadomska *et al*., 2019. Such models are designed to inform intervention and control strategies, where a minimum and significant set of contributory factors and parameters have been identified for specific niches and applied appropriately.

In closing, the projects herein represent an opportunity to launch the development of a standardised design and multi-levelled framework for studying the selection and transmission of AMR in these settings. An exciting prospect given that disparate approaches globally are a limiting factor in the progression of our understanding. As reflected above however, it is important to enable flexible and adaptable responses to the plastic nature of AMR selection and spread, and the localised factors facilitating this.

8. Contacts

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9. Chronological Publication Output of Programme

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11. Appendices

Appendix I: Co-Investigators for Research & Pump Priming Projects

[P1] EVAL Farms: Evaluating the threat of Antimicrobial Resistance in Agricultural Manures & Slurries
Dr Sujatha Raman, Prof. Theodroe Kypraios, Dr Helen West, Prof. Richard D Emes, Prof. David A Barrett, Dr Jonathan L Hobman, Prof. Christine ER Dodd, Dr Carol Morris, Dr Stephen Ramsden, Dr Andrew D Millard, Dr Jan-Ulrich Kreft, Dr Michael A Jones, Prof. Rachel L Gomes, Dr Christopher D Hudson, Prof. Christopher M Thomas
[P2] Chicken or the Egg: Is AMR in the Environment Driven by Dissemination of Antibiotics or Antibiotic Resistance Genes
Dr Katja Lehman, Dr Liz Wellington, Dr Chiara Borsetto, Dr Will Gaze, Dr Lihong Zhang, Dr Andrew Mead

[P3] Acquisition and Selection of Antibiotic Resistance in Companion and Farmed Animals and Implications for Transmission to Humans
Prof. David C Barrett, Dr Katherine M Turner, Prof. Kristen Reyher, Dr Tristan Cogan, Prof. Margaret May, Dr Severine Tasker, Dr Rachel Casey, Prof. Alasdair MacGowan
[**P4] The environmental REsistome: confluence of Human and Animal Biota in antibiotic resistance spread (REHAB)**
Dr Dan Read, Prof. Muna Anjum, Prof. Derrick Crook, Prof. Timothy Peto, Prof. Ann Walker, Dr Daniel Wilson, Dr Nicola De Maio, Dr Anna Sheppard, Prof. MJ Bailey, Dr Hyun Soon Gweon, Dr Michael Bowes, Dr Richard Smith, Prof. Neil Woodford
[P5] Occurrence and horizontal gene transfer of carbapenemase and ESBL genes in soil microbiomes
[P6] Quantifying Spatial AMR Patterns across Urban and Rural Landscapes
Dr David W Graham, Dr Martin Cooke, Dr Clare McCann, Dr Eulyn Pagaling, Dr Thomas Freitag, Dr Robert Hough, Dr Lisa Avery, Dr Yong-Guan Zhu, Dr Jian-Qiang Su
[P7] Spatial and temporal dynamics of AMR transmission from the outdoor environment to humans in urban and rural Bangladesh
Prof. Paul Wood, Michael Smith, Dr Aminul Islam, Dr Leanne Unicomb
[P8] Identification of novel double stranded RNA elements in developing antibiotic resistance in the agricultural environment
Dr Jess Rollason, Dr Lauren Acton, Dr Matthew Lamaudiere, Dr Gareth Weedall
[P9] Impact of stereochemistry of antimicrobial agents on their environmental fate, biological potency and the emergence of resistance
Prof. Ed Feil, Dr Simon Lewis
[P10] The dynamics of antimicrobial resistance gene prevalence on a commercial pig farm: implications of policy
Prof. David Gally, Prof. M Hutchings
[P11] A quantitative method to evaluate AMR distribution in complex communities based on methylome profiling
DrJose Jimenez, Prof. Gang Fang, Dr John Kenny
[P12] Genes of past, present and future: does legacy pollution contribute to antibiotic resistance in industrialised estuaries?
Dr Charles Knapp, Prof. Andrew Hursthouse, Dr Iain McLellan, Dr Roderick Williams