Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

Antibiotic resistance gene discovery in food-producing animals

Heather K Allen

Numerous environmental reservoirs contribute to the widespread antibiotic resistance problem in human pathogens. One environmental reservoir of particular importance is the intestinal bacteria of food-producing animals. In this review I examine recent discoveries of antibiotic resistance genes in agricultural animals. Two types of antibiotic resistance gene discoveries will be discussed: the use of classic microbiological and molecular techniques, such as culturing and PCR, to identify known genes not previously reported in animals; and the application of high-throughput technologies, such as metagenomics, to identify novel genes and gene transfer mechanisms. These discoveries confirm that antibiotics should be limited to prudent uses.

Addresses
Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA 50010, USA

Corresponding author: Allen, Heather K (heather.allen@ars.usda.gov)

Current Opinion in Microbiology 2014, **19**:25–29

This review comes from a themed issue on **Ecology and industrial microbiology**

Edited by **Kornelia Smalla** and **James M Tiedje**

For a complete overview see the Issue and the Editorial

Available online 1st July 2014

http://dx.doi.org/10.1016/j.mib.2014.06.001

Introduction

Bacteria harbor antibiotic resistance genes in the absence of anthropogenic selective pressure. The primary role of resistance genes in natural microbial communities could be for a function other than resistance [1]. Effects of anthropogenic antibiotic use have been to select for the movement of resistance genes onto mobile elements, the transfer of mobile elements among different bacteria (including pathogens), and the flow of resistant bacteria among environments [2••]. This has led to the definition of two eras of antibiotic resistance gene evolution: natural antibiotic resistance that occurred in microbial communities before the early 1900s, and acquired antibiotic resistance that has disseminated as a result of the selective pressure of antibiotic use [3••]. Since retrospective studies are challenging, contemporary research on antibiotic resistance genes in animal microbiomes is largely an investigation of acquired resistance traits. Animal microbiomes have acquired antibiotic resistance genes over several decades' exposure to antibiotics and heavy metals, both of which are used for treating disease, preventing disease, and improving feed efficiency. Observations of acquired resistance genes, plus the discovery of emerging resistance genes, are important to inform potential resistance problems in agricultural systems. In this sense, the emerging picture of resistance genes can be defined as both familiar resistance genes that are new to a species or ecosystem, and as novel resistance genes not previously reported.

Depth of antibiotic resistance problem *via* culturing or PCR — discovery of what we know

Classical molecular and microbiological techniques, such as PCR and culturing, continue to be important for defining the dissemination of known antibiotic resistance genes in foodborne pathogens, animal microbiomes, and the environment (Figure 1). Use of these methods to monitor antibiotic resistance genes in foodborne pathogens and commensal bacteria has been implemented in some countries, including the U.S. (NARMS [4]) and the E.U. [5]. Among seven European countries for whom antibiotic use and antibiotic resistance data were available, a clear correlation was seen between antibiotic use and resistance gene prevalence in food animals [6•]. However, a major gap in knowledge for most countries excepting Denmark [7] is data on antibiotic use in specific animal species on particular farms. Effective monitoring of bacterial antibiotic resistance requires these data to fully assess the impacts of various antibiotic types and quantities on resistance gene prevalence. An additional concern with some monitoring programs is that they are only collecting data on one aspect of antibiotic resistance — those resistance traits harbored by the organisms or genes being monitored.

Given that microbial communities are a persistent reservoir of resistance genes [8,9], basic research on emerging antibiotic resistance genes is essential to inform issues potentially left incomplete by federal monitoring programs. For example, pathogens possessing extended-spectrum beta-lactamases (ESBLs) are a world-wide clinical problem, conferring resistance to even third-generation cephalosporins and impeding bacterial disease treatment [10]. Most ESBL-producing bacteria are isolated from humans, not animals [11], but ESBL-producing *Escherichia coli* are being detected with increased frequency in animals [12•]. Recent studies have revealed that

Glossary
Animal microbiome: the microbial community associated with animals, including gut commensal bacteria
Co-selection: the indirect selection for one antibiotic resistance gene along with another resistance gene, often due to co-carriage on a mobile genetic element
Metagenomics: the collective genome of an assemblage of organisms

although transmission routes are sometimes unclear, ESBL-producing *E. coli* or *Enterobacteriaceae* can be found in the animal feed [13] and in livestock and poultry feces [13–20] in many developed countries. One important development was the PCR-based discovery of the CTX-M type ESBL in U.S. cattle, which was the first report of this type of ESBL in food-producing animals in the U.S. [19]. Additionally, these studies have shown that genes encoding ESBLs are often found on mobile genetic elements and are associated with other resistance genes. This suggests that the application of multiple types of antibiotics, not only beta-lactams, could co-select for ESBLs and lead to the persistence of ESBLs in microbial communities. Finally, ESBL-producing *E. coli* are sometimes detected in food animal products [21–23], but the routes of transmission along the food supply chain are rarely clear. Taken together, the discovery of clinically relevant antibiotic resistance genes in food-producing animals presents a paradox: bacterial antibiotic resistance genes are linked between humans and animals, but the concomitant gaps in knowledge about how those linkages are made prevent efficacious interventions.

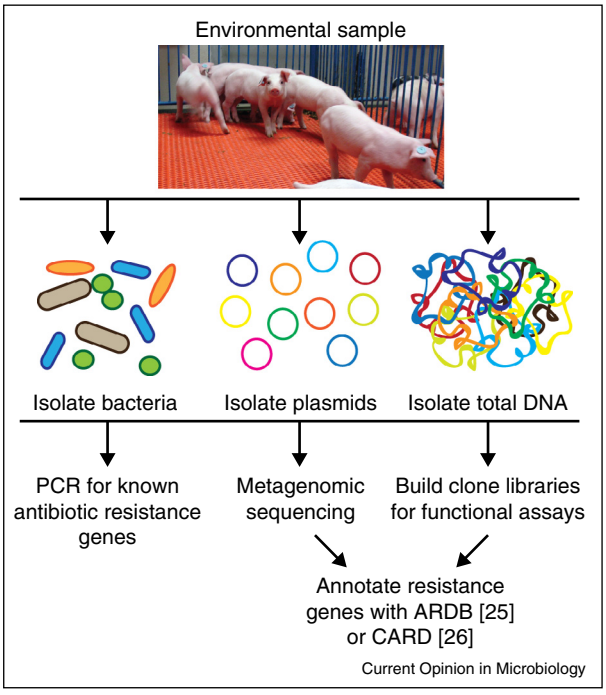
High-throughput technologies show breadth of resistance problem — discovery of what we don't know

Although PCR-based approaches are excellent for discovering homologues of known resistance genes, PCR does not enable the discovery of unknown or distantly related resistance genes. Metagenomic analysis of the total DNA from a microbial community is a powerful tool to assess the entire antibiotic resistome (Figure 1) [24]. Metagenomics based on high-throughput sequencing is suited to discover unanticipated aspects of antibiotic resistance gene ecology, including the presence of unforeseen resistance genes or changes in the abundance of related resistance genes. As with PCR-based methods, the discovery of truly novel resistance genes is limited, in this instance because metagenomics relies on homology searches to annotate DNA sequences. The annotation of antibiotic resistance genes has been improved, first by the Antibiotic Resistance gene DataBase (ARDB) [25] and more recently by the Comprehensive Antibiotic Resistance Database (CARD) [26]. The results of metagenomic analyses lend a broad view of antibiotic resistance across all environments, including animal intestinal microbiomes.

Analyses of metagenomic sequences from beef cattle feces, chicken ceca, and swine feces all reveal an abundance of resistance genes regardless of antibiotic treatment. In a study of conventionally raised beef cattle with no exposure to therapeutic antibiotics, sequence-based metagenomics predicted that 3.7% of the sequences encoded resistance to antibiotic and toxic compounds [27]. Of these genes, nearly half of them encoded multidrug resistance efflux pumps. A similar study of two chicken ceca revealed the same result [28].

The authors of the cattle study also compared the cattle fecal metagenomes to metagenomes from other agricultural systems (chicken cecum, cow rumen, and farm soil), three human fecal samples, and unrelated ecosystems (Sargasso sea and Antarctic lake), showing that the agricultural or host-associated metagenomes harbored an increased abundance in genes encoding resistance to antibiotic and toxic compounds compared to the metagenomes from remote marine environments [27]. It is important to note that the remote marine environments nonetheless harbored some of the same antibiotic resistance genes as were found in the host-associated metagenomes. Certain genes that we know to confer antibiotic resistance actually perform other functions, such as efflux pumps, in their native microbial communities [1,29,30]. Subsequent gene expression and functional analyses are

Figure 1



Routes of antibiotic resistance gene discovery are discussed in this manuscript. Both classical methods such as culturing and contemporary methods such as metagenomics inform antibiotic resistance gene ecology. Specialized databases for annotating resistance genes are the Antibiotic Resistance gene DataBase (ARDB) [25] and the Comprehensive Antibiotic Resistance Database (CARD) [26].

therefore important to determine whether annotated resistance genes can confer resistance to antibiotics. Additionally, the global dissemination of antibiotic resistance genes is apparent, even of so-called acquired resistance genes in the absence of selective pressure [31]. As the quantity of genetic data from diverse environments continues to increase, comparisons such as these will inform the breadth of antibiotic resistance gene dissemination in and among environments.

Recent analyses of swine fecal metagenomes confirm that they too harbor diverse and abundant resistance genes regardless of antibiotic treatment [32•,33•]. The effect of antibiotic treatment on antibiotic resistance has been additionally demonstrated. Zhu *et al.* showed that antibiotic treatment of swine in three commercial swine farms in China led to increased diversity of antibiotic resistance genes in manure [33•]. Looft *et al.* analyzed the effect of two weeks of continuous in-feed antibiotic treatment (ASP250 [penicillin, chlortetracycline, and sulfamethazine]) on resistance genes in two separate studies [32•,34]. Certain antibiotic resistance genes increased in abundance in the metagenomes from medicated animals. Some of these resistance genes conferred resistance to the antibiotics administered, but at least one annotated gene, an aminoglycoside O-phosphotransferase, increased in abundance despite conferring resistance to antibiotics not administered in the study (aminoglycosides) [32•]. This resistance gene has been reported on mobile elements in Gram-negative bacteria [34] and is therefore likely part of the acquired antibiotic resistome of swine gut bacteria. These results are important in light of recent US FDA guidelines for the regulation of clinically important antibiotics for use in agriculture [35] because they demonstrate that in this age of acquired resistance, a given antibiotic might select for unanticipated co-resistance or cross-resistance. Reducing the selective pressure of only certain antibiotics may not cause a reduction in resistance genes of human and animal importance [36].

One potential disadvantage of the sequence-based metagenomic approach is the detachment of genetic context, such as adjacent markers of horizontal gene transfer and indicators of the host bacterium of origin. Improved assembly of metagenomic sequences will provide genetic context *in silico*, but care must be taken to avoid conclusions drawn from misassembled sequences. Technical efforts to maintain genetic context have not been widely applied in any ecosystem but are essential for filling gaps in knowledge about resistance gene transfer in the environment. One approach is to isolate plasmids from environmental samples. For example, the isolation of IncP-1ε plasmids from pig manure and manure-amended soil indicated the broad dissemination of this group of plasmids and their associated resistance genes in this agricultural system [37•]. Experiments to determine the original host range for the plasmids suggested that they were harbored at least by culturable *Beta-proteobacteria* and *Gamma-proteobacteria* [37•]. Recent plasmid metagenomic analyses have discovered novel plasmids carrying antibiotic resistance genes in activated sludge [38] and surprising cross-phyla mosaicism in plasmids of the cow rumen [39•]. These results emphasize how much remains to be defined regarding resistance gene transfer among diverse bacteria in the environment.

An additional approach to maintaining the genetic context of antibiotic resistance genes is functional metagenomics, which is the cloning and expressing of environmental DNA in a bacterial host such as *E. coli* [40,41]. The value of functional metagenomics is theoretically limited by heterologous gene expression, and yet its use continues to detect previously unknown phenomena. This method has been applied to the intestinal microbiome of organic pigs in a study of tetracycline resistance. Numerous discoveries were made, including novel tetracycline resistance genes, known tetracycline resistance genes not previously observed outside of the human gut microbiome, and a preponderance of mobile genetic elements adjacent to most resistance genes [42••]. One tetracycline-resistant clone encoded a transposase that was related to the IS*982* and IS*4* insertion sequence families, and another was 99.7% similar to plasmid pSC101 (IncQ) from *Salmonella enterica* serovar Typhimurium [42••]. Taken together, analyses that preserve flanking DNA provide insights into how resistance genes persist and are transferred in bacterial communities.

Conclusions

Discoveries of antibiotic resistance genes in animals have revealed that their gut microbiome is a reservoir of known and novel antibiotic resistance genes, and that resistant bacteria and resistance genes are shared between human and animal microbiomes. It is important to note that although food-producing animals contribute to the broad dissemination of antibiotic resistant bacteria and genes of clinical importance [43], they are not necessarily the originator of the resistance problem [12•,44]. The original source of the gene encoding the CTX-M-5 ESBLs, for example, is the chromosome of the enteric bacterium *Kluyvera ascorbata* that was originally isolated from humans [45••]. *K. ascorbata* is a commensal bacterium of both humans and animals, and selective pressure led to the mobilization of its beta-lactamase onto a plasmid, which was then shared among commensal bacteria such as *E. coli* [45••]. Other ESBLs have emerged in parallel and disseminated through enteric bacteria of both humans and animals. All vertebrates have a largely similar intestinal microbiome at the phylum level [46], and indeed some species of bacteria (such as *E. coli*) are explicitly shared. Additionally, routes of transmitting resistant bacteria from humans to animals, and *vice versa*, need improved control to reduce the probability of exchange.

Both broad and specific methods of antibiotic resistance gene detection will continue to be important for making discoveries. For example, quantitative PCR-based tools are appropriate for determining anthropogenic impacts on resistance gene contamination in the environment [47••]. Other tools, such as functional metagenomics, are warranted to reveal the physical connections between resistance genes and mobile genetic elements [42••]. The choice of method depends on the biological question being asked, and the antibiotic resistance problem is not without biological questions in need of being addressed.

Continued antibiotic selective pressure in both humans and animals fuel the dissemination of acquired resistance once genes have been mobilized. Antibiotics are the primary defense against bacterial disease in both human and veterinary medicine; antibiotics need to be used more prudently in both human and veterinary medicine in order to slow down resistance gene distribution and prevent the emergence of new resistance genes.

Acknowledgements

I am indebted to Lisa Durso, Torey Looft, and Thad Stanton for feedback on early drafts of the manuscript. Also, I appreciate NADC librarian Jennifer Muller for acquiring some of the reference materials. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J: **Call of the wild: antibiotic resistance genes in natural environments**. *Nat Rev Microbiol* 2010, **8**:251-259.

2. •• Stokes HW, Gillings MR: **Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens**. *FEMS Microbiol Rev* 2011, **35**:790-819.
This review is both thorough and clear in its presentation of antibiotic resistance gene dissemination at the gene and bacterium levels.

3. •• Martinez JL: **Natural antibiotic resistance and contamination by antibiotic resistance determinants: the two ages in the evolution of resistance to antimicrobials**. *Front Microbiol* 2012, **3** http://dx.doi.org/10.3389/fmicb.2012.00001.
This review is a concise presentation of what can be a difficult concept to explain, which is the evolutionary difference between naturally occurring and acquired antibiotic resistance genes, and why this difference is significant.

4. White D, Fedorka-Cray P, Chiller C: **The national antimicrobial resistance monitoring system (NARMS)**. In *National Mastitis Council Annual Meeting Proceedings*. 2006:56-60.

5. Silley P, de Jong A, Simjee S, Thomas V: **Harmonisation of resistance monitoring programmes in veterinary medicine: an urgent need in the EU?** *Int J Antimicrob Agent* 2011, **37**:504-512.

6. • Chantziaras I, Boyen F, Callens B, Dewulf J: **Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries**. *J Antimicrob Chemother* 2014, **69**:827-834.
One of the few studies to date that correlates antibiotic use with antibiotic resistance at a country-wide level.

7. Stege H, Bager F, Jacobsen E, Thougaard A: **VETSTAT — the Danish system for surveillance of the veterinary use of drugs for production animals**. *Prev Vet Med* 2003, **57**:105-115.

8. Salyers AA, Gupta A, Wang Y: **Human intestinal bacteria as reservoirs for antibiotic resistance genes**. *Trends Microbiol* 2004, **12**:412-416.

9. Sommer MO, Dantas G: **Antibiotics and the resistant microbiome**. *Curr Opin Microbiol* 2011, **14**:556-563.

10. Paterson DL, Bonomo RA: **Extended-spectrum β-lactamases: a clinical update**. *Clin Microbiol Rev* 2005, **18**:657-686.

11. Carattoli A: **Animal reservoirs for extended spectrum β-lactamase producers**. *Clin Microbiol Infect* 2008, **14**:117-123.

12. • Hunter PA, Dawson S, French GL, Goossens H, Hawkey PM, Kuijper EJ, Nathwani D, Taylor DJ, Teale CJ, Warren RE: **Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies**. *J Antimicrob Chemother* 2010, **65**:i3-i17.
This review uniquely covers both the human and veterinary medical perspectives on three antibiotic-resistant pathogens (methicillin-resistant *Staphylococcus aureus*, extended-spectrum beta-lactamase-producing bacteria, and *Clostridium difficile*), additionally indicating gaps in knowledge in multiple places.

13. Rodrigues C, Machado E, Peixe L, Novais A: **IncI1/ST3 and IncN/ST1 plasmids drive the spread of *bla*TEM-52 and *bla*CTX-M-1/-32 in diverse *Escherichia coli* clones from different piggeries**. *J Antimicrob Chemother* 2013, **68**:2245-2248.

14. Gonçalves A, Torres C, Silva N, Carneiro C, Radhouani H, Coelho C, Araújo C, Rodrigues J, Vinué L, Somalo S: **Genetic characterization of extended-spectrum beta-lactamases in *Escherichia coli* isolates of pigs from a Portuguese intensive swine farm**. *Foodborne Pathog Dis* 2010, **7**:1569-1573.

15. Schmid A, Hörmansdorfer S, Messelhäusser U, Käsbohrer A, Sauter-Louis C, Mansfeld R: **Prevalence of extended-spectrum β-lactamase-producing *Escherichia coli* on Bavarian dairy and beef cattle farms**. *Appl Environ Microb* 2013, **79**:3027-3032.

16. Chauvin C, Le Devendec L, Jouy E, Le Cornec M, Francart S, Marois-Créhan C, Kempf I: **National prevalence of resistance to third-generation cephalosporins in *Escherichia coli* isolates from layer flocks in France**. *Antimicrob Agents Chemother* 2013, **57**:6351-6353.

17. Geser N, Stephan R, Kuhnert P, Zbinden R, Kaeppeli U, Cernela N, Haechler H: **Fecal carriage of extended-spectrum-lactamase-producing *Enterobacteriaceae* in swine and cattle at slaughter in Switzerland**. *J Food Prot* 2011, **74**:446-449.

18. Ramos S, Silva N, Dias D, Sousa M, Capelo-Martinez JL, Brito F, Caniça M, Igrejas G, Poeta P: **Clonal diversity of ESBL-producing *Escherichia coli* in pigs at slaughter level in Portugal**. *Foodborne Pathog Dis* 2013, **10**:74-79.

19. Wittum TE, Mollenkopf DF, Daniels JB, Parkinson AE, Mathews JL, Fry PR, Abley MJ, Gebreyes WA: **CTX-M-type extended-spectrum β-lactamases present in *Escherichia coli* from the feces of cattle in Ohio, United States**. *Foodborne Pathog Dis* 2010, **7**:1575-1579.

20. Hiroi M, Yamazaki F, Harada T, Takahashi N, Iida N, Noda Y, Yagi M, Nishio T, Kanda T, Kawamori F: **Prevalence of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in food-producing animals**. *J Vet Med Sci* 2012, **74**:189-195.

21. Geser N, Stephan R, Hächler H: **Occurrence and characteristics of extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* in food producing animals, minced meat and raw milk**. *BMC Vet Res* 2012, **8**:21.

22. Jensen LB, Hasman H, Agersø Y, Emborg H-D, Aarestrup FM: **First description of an oxyimino-cephalosporin-resistant, ESBL-carrying *Escherichia coli* isolated from meat sold in Denmark**. *J Antimicrob Chemother* 2006, **57**:793-794.

23. Lavilla S, Gonzalez-Lopez J, Miro E, Dominguez A, Llagostera M, Bartolome R, Mirelis B, Navarro F, Prats G: **Dissemination of extended-spectrum β-lactamase-producing bacteria: the**

food-borne outbreak lesson. *J Antimicrob Chemother* 2008, **61**:1244-1251.

24. D'Costa VM, McGrann KM, Hughes DW, Wright GD: **Sampling the antibiotic resistome**. *Science* 2006, **311**:374-377.

25. Liu B, Pop M: **ARDB — Antibiotic Resistance genes DataBase**. *Nucleic Acids Res* 2009, **37**:D443-D447.

26. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Ejim L: **The comprehensive antibiotic resistance database**. *Antimicrob Agents Chemother* 2013, **57**:3348-3357.

27. Durso LM, Harhay GP, Bono JL, Smith TP: **Virulence-associated and antibiotic resistance genes of microbial populations in cattle feces analyzed using a metagenomic approach**. *J Microbiol Method* 2011, **84**:278-282.

28. Qu A, Brulc JM, Wilson MK, Law BF, Theoret JR, Joens LA, Konkel ME, Angly F, Dinsdale EA, Edwards RA: **Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome**. *PLoS ONE* 2008, **3**:e2945.

29. Martinez JL, Sánchez MB, Martínez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C: **Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems**. *FEMS Microbiol Rev* 2009, **33**:430-449.

30. Dantas G, Sommer MO: **Context matters — the complex interplay between resistome genotypes and resistance phenotypes**. *Curr Opin Microbiol* 2012, **15**:577-582.

31. Pallecchi L, Bartoloni A, Paradisi F, Rossolini GM: **Antibiotic resistance in the absence of antimicrobial use: mechanisms and implications**. *Expert Rev Anti Infect Ther* 2008, **6**:725-732.

32. • Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, Sul WJ, Stedtfeld TM, Chai B, Cole JR *et al.*: **In-feed antibiotic effects on the swine intestinal microbiome**. *Proc Natl Acad Sci U S A* 2012, **109**:1691-1696.
Both metagenomic and PCR-based approaches were used in this study to describe the depth and breadth of antibiotic resistance gene diversity in swine feces, with the results suggesting co-selection of resistance genes unrelated to the antibiotics administered.

33. • Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD, Hashsham SA, Tiedje JM: **Diverse and abundant antibiotic resistance genes in Chinese swine farms**. *Proc Natl Acad Sci U S A* 2013, **110**:3435-3440.
This is an impressive study using highly parallel quantitative PCR to investigate resistance gene abundance in swine manure, manure compost, and manure-amended soil from commercial farms in China, thus informing the fate of fecal antibiotic resistance genes on a large scale.

34. Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP, Henrissat B, Stanton TB: **Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations**. *ISME J* 2014 http://dx.doi.org/10.1038/ismej.2014.12. (Epub ahead of print).

35. US Department of Health and Human Services, Food and Drug Administration, Center for Veterinary Medicine: *The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals*. Guidance for Industry #209; 2012:: 1-26.

36. O'Brien TF: **Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else**. *Clin Infect Dis* 2002, **34**:S78-S84.

37. • Heuer H, Binh CTT, Jechalke S, Kopmann C, Zimmerling U, Krögerrecklenfort E, Ledger T, González B, Top EM, Smalla K: **IncP-1ε plasmids are important vectors of antibiotic resistance genes in agricultural systems: diversification driven by class 1 integron gene cassettes**. *Front Microbiol* 2012, **3** http://dx.doi.org/10.3389/fmicb.2012.00001.
In addition to the isolation and study of IncP-1 group plasmids and their associated resistance genes, the original bacterial hosts were identified empirically.

38. Zhang T, Zhang X-X, Ye L: **Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge**. *PLoS ONE* 2011, **6**:e26041.

39. • Kav AB, Sasson G, Jami E, Doron-Faigenboim A, Benhar I, Mizrahi I: **Insights into the bovine rumen plasmidome**. *Proc Natl Acad Sci U S A* 2012, **109**:5452-5457.
This is a method development of isolating and sequencing environmental plasmids in bulk (the plasmidome), which could be an exciting application for antibiotic resistance gene research.

40. Schmieder R, Edwards R: **Insights into antibiotic resistance through metagenomic approaches**. *Future Microbiol* 2012, **7**:73-89.

41. Handelsman J: **Metagenomics: application of genomics to uncultured microorganisms**. *Microbiol Mol Biol Rev* 2004, **68**:669-685.

42. •• Kazimierczak KA, Scott KP, Kelly D, Aminov RI: **Tetracycline resistome of the organic pig gut**. *Appl Environ Microb* 2009, **75**:1717-1722.
The application of functional metagenomics in this study revealed unexpected resistance genes, and mobile genetic elements that could contribute to the persistence of resistance in organic pigs.

43. Marshall BM, Levy SB: **Food animals and antimicrobials: impacts on human health**. *Clin Microbiol Rev* 2011, **24**:718-733.

44. Singer RS, Finch R, Wegener HC, Bywater R, Walters J, Lipsitch M: **Antibiotic resistance — the interplay between antibiotic use in animals and human beings**. *Lancet Infect Dis* 2003, **3**:47-51.

45. •• Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A: **β-Lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types**. *Antimicrob Agents Chemother* 2002, **46**:3045-3049.
This is one of the few reports of the origin of a plasmid-borne antibiotic resistance gene in pathogens; in this case the source is a bacterium found in the human microbiota, thereby serving as a reminder that the environmental reservoir of antibiotic resistance genes is not always remote.

46. Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI: **Worlds within worlds: evolution of the vertebrate gut microbiota**. *Nat Rev Microbiol* 2008, **6**:776-788.

47. •• Pruden A, Arabi M, Storteboom HN: **Correlation between upstream human activities and riverine antibiotic resistance genes**. *Environ Sci Technol* 2012, **46**:11541-11549.
This is an outstanding application of quantitative PCR for antibiotic resistance genes across a watershed to demonstrate anthropogenic impacts on antibiotic resistance gene abundance.