METHOD ARTICLE

Plasmidome AMR screening (PAMRS) workflow: a rapid screening workflow for phenotypic characterization of antibiotic resistance in plasmidomes [version 1; peer review: 1 approved with reservations]

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Abstract

Background: Phenotypic characterization of antimicrobial resistance (AMR) in bacteria has remained the gold standard for investigation and monitoring of what resistance is present in an organism.

However, the process is laborious and not attractive for screening multiple plasmids from a microbial community (plasmidomes). Instead, genomic tools are used, but a major bottle neck that presence of genes does not always translate into phenotypes.

Methods: We designed the plasmidome AMR screening (PAMRS) workflow to investigate the presence of antibiotic resistant phenotypes in a plasmidome using *Escherichia coli* as a host organism. Plasmidomes were extracted from the faecal matter of chicken, cattle and humans using commercial plasmid extraction kits. Competent *E. coli* cells were transformed and evaluated using disk diffusion. Thirteen antibiotic resistant phenotypes were screened.

Results: Here, we show that multiple antibiotic resistant phenotypes encoded by plasmids can be rapidly screened simultaneously using the PAMRS workflow. *E. coli* was able to pick up to 7, 5 or 8 resistant phenotypes from a single plasmidome from chicken, cattle or humans, respectively. Resistance to ceftazidime was the most frequently picked up phenotype in humans (52.6%) and cattle (90.5%), whereas in chickens, the most picked up resistant phenotype was resistance to co-trimoxazole, ceftriaxone and ampicillin (18.4% each).

Conclusions: This workflow is a novel tool that could facilitate studies to evaluate the occurrence and expression of plasmid-encoded antibiotic resistance in microbial communities and their associated plasmid-host ranges. It could find application in the screening of

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Any reports and responses or comments on the article can be found at the end of the article.

plasmid-encoded virulence genes.

Keywords

Antibiotic Resistance, Plasmidomes, Plasmids, Rapid Screening, AMR Phenotypes

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Introduction

Antibiotic-resistant bacterial infections are a major public health problem. Annually, some estimated 30,000 deaths are recorded in the EU alone¹, while the morbidity and mortality in low to middle income countries in South America, Asia and Africa are even higher²⁻⁴. Generally, bacteria develop mechanisms to either prevent antibiotics from reaching toxic levels inside the cell, modify the targets of the antibiotic or degrade the antibiotic itself⁵. These drug evasion mechanisms are normally developed either through gene mutations or acquisition of antibiotic resistance genes (ARGs) from other bacteria through horizontal gene transfer (HGT), a process that contributes significantly to the spread of ARGs⁶. While HGT can occur in any environment, it is more common in niches within the soil⁷, wastewater treatment plants^{8,9}, and the gut microbiome of humans and animals¹⁰⁻¹².

Transformation of bacteria with naked DNA from the extracellular environments is a major contributor to HGT and the spread of ARGs¹³. Interestingly, over 80 bacterial species can be transformed due to the presence of genes involved in DNA uptake, suggesting that, this trait of competency is widespread¹⁴. While it is not clear what stimuli enhance the transformation of bacteria, the absence of nutrients and the presence of competence-inducing peptides have been identified as triggers¹⁵. Although DNase degrades most gut DNA¹⁶, intact plasmids have been isolated from the gut contents of plasmid-fed rodents, suggesting that environmental or extracellular DNA are taken up by naturally occurring competent bacteria¹³. Data suggest that *E. coli* can be transformed with extracellular plasmid DNA under natural conditions^{17,18}, and these observations suggest that bacteria could be transformed by DNA in the gut and may contribute to the spread of ARGs.

Some bacterial plasmids have been shown to carry mobile genetic elements that can confer resistance to a variety of antimicrobials, including last line¹⁹⁻²³. It has been found that large conjugative resistance plasmids follow the same evolutionary trajectories as their non-conjugative mini-replicons in the same and other species¹⁹. In addition plasmids harbouring multiple antimicrobial-resistance determinants (R-plasmids) can be transferred in simulated natural microenvironments from various bacterial pathogens of human, animal, or fish origin to susceptible strains isolated from a different ecological niche²⁴.

Traditionally, detection of phenotypes of antibiotic resistance has been performed through culture-based techniques. To understand the role of plasmids in spreading antibiotic resistance, efforts have centred around polymerase chain reaction (PCR) amplification of targets or sequencing. Although sequencing and PCR approaches provide a high resolution of AMR investigation, they are limited by their inability to determine which genetic elements are expressed and which ones are not, hence the need for phenotypic tools to complement such metagenomic or plasmidome sequencing approaches. To determine the burden of active or expressed resistance genes in human, chicken, and cattle gut microbiomes, we developed the Plasmidome AMR screening (PAMRS) workflow using a combination of plasmidome isolation, bacterial transformation, and multi-disk diffusion to rapidly investigate the ecology of thirteen (13) antibiotic resistant phenotypes from gut plasmidomes using *Escherichia coli* as a host.

Methods

Development of the workflow

The workflow integrates the well-established protocols for plasmid extraction, bacteria transformation and antimicrobial susceptibility testing by disk diffusion. We adopted *E. coli JM109* as host for our experiments. The BioBrick plasmids pSB1C3, pSB1A3, pSB1K3 and pSB1T3 containing the red fluorescent protein (J04450) expression cassette and encoded by resistance genes for chloramphenicol, ampicillin, kanamycin, and tetracycline respectively (<http://parts.igem.org/Catalog>) were used as plasmids to evaluate the workflow. The plasmid backbones for all the four plasmids are similar with the differences being the type of antibiotic resistance carried (Figure 1). The replication origin is a pUC19-derived pMB1 which is permissive in *E. coli* and some other bacteria.

Transformation of cells

Chemically competent *E. coli JM109* cells were made using the TSS Buffer protocol as described previously²⁵ with modifications. Briefly, 2μL of plasmid was added to 100 μL of competent cells and incubated on ice for 45 minutes. The cells were heat shocked at 42°C for 90 seconds and the tubes were returned to ice for 90 seconds. Following incubation on ice, 0.9mL Luria broth was added and the cells were conditioned at 37 °C for 1 hour. Recombinant clones were identified by inoculating 100 μL of the conditioned cells on Luria Agar incorporated with

Figure 1. Plasmid maps of control plasmids used for evaluation of the workflow. Plasmid maps generated by the LabGenius BioBrick Mapper and downloaded from the Registry of Standard Biological Parts (parts.igem.org).

appropriate antibiotics (i.e. 40 µg/µl chloramphenicol; carbenicillin 80 µg/µl; kanamycin 40 µg/µl and tetracycline 5 µg/µl) (Merck KGaA, St. Louis, USA). As an alternative to incorporation of antibiotics into the agar, antibiotic disks with the respective antibiotics were evaluated. To do this, the disks were placed onto the agar plates without antibiotics incorporated into them. The inoculated plates with either antibiotics incorporated within, or antibiotic disks (Mast Group Ltd, Bootle, UK) placed on the surface was incubated at 37°C overnight. Recovery of recombinant clones was compared between plates with antibiotics incorporated and those with antibiotic disks placed on the surface. Following experiments with individual plasmids, combinations of all four were made and evaluated as described above. First, equal concentrations of plasmids (1:1:1:1) was evaluated and then varying ratios (1:3:5:10) for the four antibiotics (interchangeably) was also evaluated.

Evaluation of the workflow with gut plasmidomes from humans, cattle, and chickens

We conducted a cross-sectional evaluation of gut plasmidomes from humans, cattle and chickens within the Ho Municipal area in the Volta Region, Ghana. The chicken specimens included broilers, old layers, cockerels, and local free-range hens and roosters purchased from markets, and street and community vendors. The chickens were obtained alive and sacrificed by slicing the jugular veins with a sharp knife. Thirty-eight (38) chickens were used. Faecal material was harvested from the caeca of the chicken. Faecal material was also sampled from the rectum of slaughtered cattle at the Ho abattoir. Twenty one (21) cattle specimen were obtained. We also collected the faecal material from volunteers in 15 randomly selected households within the Ho municipal area. A household was defined as one or several persons who live in the same dwelling and share meals. The households were not selected in a particular format. The study team knocked on doors to introduce the study and those that consented were included. Volunteers were handed sterile stool containers to submit their stool samples, after written inform consent had been obtained. The faecal samples were kept at 4°C and processed within 24 hours.

Plasmidome extraction and transformation

A loopful of each faecal sample was picked using a sterile 10 µL microbiological loop and inoculated into 5 mL of brain heart infusion broth (BHIB) (Oxoid, UK). The broth was cultured at 37°C with shaking at 200 rpm overnight. Plasmidome extraction was performed using the QIAprep Spin Miniprep Kit (Qiagen GmbH, Germany) following the manufacturer's protocol. Briefly, the cells were harvested by centrifuging 1.8 mL of culture in a 2 mL microcentrifuge tube at 7,000xg for 15 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended and lysed. Plasmid DNA (pDNA) was purified by binding to the spin columns. The columns were washed and the pDNA was eluted with 50 µL buffer EB. Competent *E. coli* cells were transformed with the extracted plasmidomes as described above and screened.

Escherichia coli JM109 was used as host for screening of the plasmidomes. *E. coli* was chosen because it is an important member of the gut microbiome as well as the antibiotic resistance story and often used an indicator or proxy organism. Chemically competent *E. coli* JM109 cells were prepared using TSS buffer (pH 6.5) and then transformed²⁵ with the extracted plasmidome. Briefly, 2 µL of extracted plasmidome was added to 100 µL of competent cells and incubated on ice for 45 minutes. Although the original publication doesn't recommend a heat shock, we included a heat shot based on our observation that helps improve transformation efficiency in a number of cases. Heat shock was performed at 42°C for 90 seconds and then the tubes were returned to ice for 90 seconds before adding 0.9 mL Luria broth. Cells were conditioned by incubating at 37°C for 1 hour.

Antibiotic screening

To screen for susceptibility to multiple antibiotics, we employed the disk diffusion method. Mueller Hinton Agar 2 (MHA) (Merck KGaA, Germany) was prepared and poured according to the manufacturer's instructions and allowed to dry. Plates were then inoculated with 100 µL of the transformed cells using sterile glass balls. The plates were left to dry in a laminar flow cabinet after which up to six antibiotic disks (Mast Group, Bootle, UK; Table 1) were applied using a manual disk stamper. The plates were incubated at 37°C, overnight. About three colonies growing in the zone of clearing was picked and subcultured on a fresh MHA plate, overnight. A single colony from the overnight plate was picked and checked to confirm

Table 1. List of antibiotics and concentrations of disks used for screening.

Code	Name	Concentration
CT 10	Colistin Sulphate	10 µg
CIP 5	Ciprofloxacin	5 µg
TET 30	Tetracyclin	30 µg
TS 25	Trimethoprim/Sulfamethoxazole (Cotrimoxazole)	1.25-23.75 µg
MEM 10	Meropenem	10 µg
CRO 30	Ceftriaxone	30 µg
AMP 10	Ampicillin	10 µg
TN 10	Tobramycin	10 µg
PTZ 30	Piperacillin/Tazobactam	30-6 µg
AUG 30	Amoxicillin/Clavulanic Acid	20-10 µg
CAZ 10	Ceftazidime	10 µg
GM 10	Gentamicin	10 µg
C 30	Chloramphenicol	30 µg

the antibiotic resistance. Confirmation of resistance was performed by repeating the screening experiment with the recombinant *E. coli* JM109 that was picked.

Data analysis

Data was entered into Microsoft Excel (Office 365 version), coded (0=no resistance; 1=resistance), analysed and summarized. Summaries were visualised as graphs in Excel.

Ethics

The study was approved by the Research Ethics Committee of the University of Health and Allied Sciences with Protocol Identification Number UHAS-REC A.9 [1] 18–19. Written informed consent was sought from participants before recruitment into the study.

Results

Evaluation of the use of antibiotic disks to screen transformants

Traditionally, transformants are screened on plates by incorporating the antibiotic into the agar and poured (Figure 2, A1 to A4). However, to enable rapid and cheaper screening of multiple resistant phenotypes we designed and evaluated experiments with antibiotic disks (Figure 2, C1 to C4). Using antibiotic disks was found to be suitable for selecting clones just like incorporating antibiotics into the agar (Figure 2, Rows A and C). When the four plasmids were combined, almost the same number of colonies were recovered irrespective of the antibiotic that was incorporated into the medium for selection (Figure 3, A1 to A4). In contrast to screening for one antibiotic at a time by incorporation of that antibiotic into the agar, all four

Figure 2. Demonstration of the use of disks for selection of antibiotic resistant transformants. The BioBrick plasmids pSB1T3, pSB1A3, pSB1C3 and pSB1K4 expressing the red fluorescent protein cassette (J04450) and conferring resistance to tetracycline, ampicillin, chloramphenicol, and kanamycin respectively were used. Competent *E. coli* JM109 cells were transformed with each plasmid and evaluated in plates incorporated with the respective antibiotics (A1 to A4) and on plates with antibiotic disks (C1 to C4). Plates B1 to B4 are plates with antibiotics inoculated with the untransformed cells demonstrating that the cells used for transformations did not already have resistance to any of the antibiotics used. A1 to A4 shows resistant clones are recovered when selection is done by incorporating antibiotics in the plates and this was similar when antibiotic disks were used (C1 to C4).

Figure 3. Demonstration of the use of disks for screening plasmidomes with multiple antibiotic resistant plasmids. The four BioBrick plasmids pSB1T3, pSB1A3, pSB1C3 and pSB1K4 were combined in equal and varying proportions to form plasmidomes. Competent *E. coli* JM109 cells were transformed with the plasmidome and evaluated in plates incorporated with individual antibiotics (A1 to A4 and C1 to C4) and on plates with multiple antibiotic disks (B1, B2 and D1 to D4). On plates that were incorporated with all four antibiotics, very few colonies were recovered (B3 and B4). Row B is plates with antibiotics and just the untransformed cells showing the cells did not already have resistance to any of the antibiotics used. Whether plasmids were combined in equal proportions (B1 and B2) or not (D1 to D4), resistant clones for all four could be picked up.

antibiotics could be screened at once on a single plate by using discs (Figure 3, B1 and B2). Few transformants picked up all four antibiotics (Figure 3, B3 and B4). When the four plasmids were combined in different ratios, colonies resistant to all four antibiotics were formed for all plasmids irrespective of the ratios used (Figure 3, C1 to C4 and D1 to D4).

Demonstration of the PAMRS workflow with human, chicken and cattle gut plasmidomes

Having demonstrated that antibiotic disks could be used to rapidly screen for multiple antibiotic resistant phenotypes at once on a single plate, we further tested the PAMRS workflow (Figure 4) on plasmidomes extracted from faecal specimens

Bacteria community enrichment and sample preparation for plasmid extraction (Stage 1)

Plasmidome Extraction with QIAprep Spin Miniprep Kit (Stage 2)

Transformation of the Screening Host Bacteria (e.g. *E. coli*) (Stage 3)

Figure 4. Overview of the PAMRS workflow. The workflow is broken down into 3 stages. The first stage comprises, sample acquisition and processing for plasmidome extraction. The processing involves enrichment of bacteria in nutrient rich medium to ensure there is adequate bacteria and plasmids to extract. Since faeces are disposed into the environment, focus on this workflow is on aerobic culture as would normally be in the environment. The second stage is plasmidome extraction using a commercial plasmid extraction kit. Competent cells are then transformed in stage three with the plasmidome extracted in stage two.

from chickens, cattle and humans. Thirteen antibiotics were screened. The cells used for transformation was not resistant to any of the antibiotics (Figure 5, A and B). Cells that pick up resistant plasmids were seen to grow in the zone of clearing as single colonies (Figure 5, C and D). For a plasmidome that did not contain resistant plasmids, clear zones were seen

(Figure 5E). The colonies on initial plates were confirmed successfully when plated on fresh plates with the same antibiotic disk they were initially resistant to (Figure 5F).

A total of 38, 21 and 19 plasmidomes from chickens, cattle and humans, respectively, were screened. Of these, samples

Figure 5. Plasmidomes from chicken, cattle and human guts were successfully screened for the presence of antibiotic resistant plasmids. Controls were plates inoculated with the wild type cells (A and B). Plasmid encoded antibiotic resistance were identified by the presence of isolated colonies in the zone of clearing for a particular antibiotic disk (C and D) whereas for samples without plasmid encoded antibiotic resistance, there were no colonies in any of the zones of clearing (E). Resistant colonies were confirmed by replating onto fresh plates with the same antibiotic they were found to be resistant to (F).

from humans were found to contain plasmids conferring resistance to all 13 antibiotics screened followed by chickens and then cattle that had plasmids conferring resistance to 12 and 10 antibiotics respectively (Figure 6A). In the humans, resistance to ceftazidime was highest (52.6%) followed by chloramphenicol, amoxicillin-clavulanic acid and ampicillin (31.6% each), tetracycline (26.3%), tobramycin, ceftriaxone and colistin sulphate (21.1%), gentamicin, meropenem and trimethoprim-sulfamethoxazole (15.8% each) and ciprofloxacin and piperacillin-tazobactam (5.3% each). In the cattle, there was no resistance to ciprofloxacin, gentamicin and amoxicillin-tazobactam. Almost all the cattle (90.5%) had plasmids conferring resistance to ceftazidime. In addition, resistance to colistin sulphate, ampicillin, trimethoprim-sulfamethoxazole, tetracycline, ceftriaxone, chloramphenicol, piperacillin-tazobactam, meropenem and tobramycin in the cattle were 33.3%, 33.3%, 23.8%, 23.8%, 19.0%, 19.0%, 14.3%, 14.3%, and 14.3% respectively. Of the chickens, the highest resistance was recorded for

ampicillin, trimethoprim-sulfamethoxazole and ceftriaxone (18.4% each), chloramphenicol and gentamicin (15.8% each), ceftazidime, colistin sulphate, piperacillin-tazobactam, amoxicillin-clavulanic acid (13.2% each), tetracycline and tobramycin (10.5% each) and meropenem 7.9%. There was no plasmid conferring resistance to ciprofloxacin in chickens. Furthermore, among the chickens, there was no resistance from broilers and the local free-range chickens. Resistance in chickens was mainly in the layers and the roosters raised in poultry farms.

Of all the samples screened, 36.8% and 31.6% of chickens and humans did not have any antibiotic resistant conferring plasmids (Figure 6B). Furthermore, 21.1%, 19.0% and 15.8% of chickens, cattle and humans had plasmids conferring antibiotic resistance to only one antibiotic. The highest number of resistance conferred in one particular sample was 8 in humans (5.3%), 5 in cattle (9.5%) and 7 in chickens (2.6%). In humans however, resistance to 5 antibiotics was found in 21.1% of the subjects

Figure 6. Prevalence of antibiotic resistant conferring plasmids in chickens, cattle, and humans. The prevalence of resistance to individual antibiotics conferred by plasmids showed a higher diversity in humans, followed by chickens and then cattle (A). The highest number of resistance conferred by plasmids from a single sample in humans, chickens and cattle was 8, 7 and 5 respectively (B).

followed by 6 antibiotics (10.5%) and 5.5% each for 2, 4 and 7, respectively. Similarly, in the chickens, resistance to 2, 3, 4, 5, and 6 antibiotics in the same sample was recorded in 15.8%, 7.9%, 7.9%, 5.3%, and 2.6% respectively. Within the cattle, the highest number of antibiotic resistance recorded in a single sample was 3 (28.6%), followed by 4 (23.8%) and two (19.0%) (Figure 6B).

Discussion

The ecological impacts of plasmids are without doubt. Plasmids carry genes responsible for producing products known to help their prokaryotic hosts compete and survive better, as well as remain flexible and adaptable in the environment. Some of these plasmid-encoded characteristics include antibiotic resistance, production of antimicrobials, degradation of xenobiotics, among others^{26,27}. Interrogation of plasmid functions have long been studied^{28–30}. These studies are based on the ability of plasmids to confer selectable markers to their hosts. With genomic tools, we have come to understand that similar to prokaryotic hosts, plasmids are also defined by ecological niches^{31,32}. However, the presence of genes does not always translate to a biological cause of phenotypes³³. Hence, using associated genetic markers can lead to incorrect predictions of functionality and the associated biological role organisms or communities.

One advantage of genomic approaches is that they give a bigger picture and appears to provide rapid answers to questions. However, since associations between genetic markers and phenotypes can be spurious³⁴, we sought to develop protocols that can be used in parallel with genomic tools to interrogate the actual role and functionality of plasmids in microbial communities. In this study, we demonstrated this with antibiotic resistant functions of plasmids. We have shown that with a combination of tools that are not traditionally used together it is feasible to rapidly investigate plasmid-encoded antibiotic resistance phenotypes. The protocol we developed and evaluated comprised extraction of the total plasmid community (the plasmidome) from a sample, transformation of a host bacteria of interest with the extracted plasmidome and then screening for the presence of the phenotypes. Studies on plasmidomes so far have largely been sequencing and bioinformatics based^{35,36} and can be complemented by phenotypic studies such as what we have demonstrated to obtain deeper insights into what genes are functional and those that are not. The system we have demonstrated can particularly be targeted to investigate the importance of plasmids in the dissemination of antibiotic resistance in medically important pathogens such as the glass priority pathogens³⁷. A targeted evaluation of plasmidomes can reveal unique adaptations employed by microbial communities

in medical facilities, human and animal guts and the environment as they respond to gradual exposure to antibiotics.

From the samples we evaluated with this method, we observed that *E. coli*, a very important pathogen and indicator organism can pick up resistant phenotypes from chicken, cattle and human faecal samples. This has many implications for the control of antibiotic resistance and suggests that the environment (e.g. soil and water) where excreta from animals and humans intersect is an important sink for evolution and spread of antibiotic resistant phenotypes encoded by plasmids. This interaction can be further exacerbated with the formation of biofilms which provide a protected environment for microbes to interact and share mobile genetic elements^{38–40}. Another key finding from this study is that humans compared to chickens and cattle appear to harbour more antibiotic resistant plasmids. Further studies in this area are required to enable the quantification of the contribution of antibiotic resistant plasmids from humans to the overall antibiotic resistant burden. Overall, the plasmidomes from the three species studies were found to harbour plasmids conferring resistance to multiple antibiotics. Resistance to ceftazidime was highest in humans and cattle. This is particularly worrying as it is a drug for treating the difficult to manage difficult to treat infections by *Pseudomonas aeruginosa*⁴¹ and other organisms. It has been demonstrated that, mutation-driven evolution of resistance to ceftazidime in *Pseudomonas aeruginosa* could develop in a span of just 30 days of exposure to increasing concentrations of the drug⁴². With the very high levels of resistance from the cattle and human samples, there is the need to review the use of the drug in these populations as well as other areas. Overall, it was evident that at least one plasmidome contained a plasmid that confers resistance to each of the thirteen antibiotics investigated. This affirms the calls for action to halt the antibiotic resistant menace and the need to investigate the mechanisms of evolution and spread

of resistance so as to develop sustainable ways to stop the spread.

Conclusion

This work has demonstrated that, antibiotic resistant phenotypes conferred by plasmidomes can be rapidly screened with phenotypic tools like the PAMRS workflow (Figure 4). The application of these techniques will enable assessment of expressed drug resistant phenotypes which otherwise cannot be determined by genome sequencing and assembly of plasmids. The tool described here can be applied to the study of other plasmidome functions such as virulence genes, among others. Data from this study suggests that humans may carry plasmids conferring resistance to more antibiotics compared to chickens and cattle. This however needs to be studied within a larger study population.

Data availability

Underlying data

Harvard Dataverse: Replication Data for PAMRS Workflow: A rapid screening workflow for phenotypic characterization of antibiotic resistance in plasmidomes, <https://doi.org/10.7910/DVN/0QZJQZ>⁴³.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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References

- Cassini A, Högberg LD, Plachouras D, et al.: **Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis.** *Lancet Infect Dis.* 2019; **19**(1): 56–66.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Stewardson AJ, Marimuthu K, Sengupta S, et al.: **Effect of carbapenem resistance on outcomes of bloodstream infection caused by Enterobacteriaceae in low-income and middle-income countries (PANORAMA): a multinational prospective cohort study.** *Lancet Infect Dis.* 2019; **19**(6): 601–10.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Lim C, Takahashi E, Hongsuwan M, et al.: **Epidemiology and burden of multidrug-resistant bacterial infection in a developing country.** *eLife.* 2016; **5**: e18082.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Gandra S, Tseng KK, Arora A, et al.: **The Mortality Burden of Multidrug-resistant Pathogens in India: A Retrospective, Observational Study.** *Clin Infect Dis.* 2019; **69**(4): 563–70.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Blair JMA, Webber MA, Baylay AJ, et al.: **Molecular mechanisms of antibiotic resistance.** *Nat Rev Microbiol.* 2015; **13**(1): 42–51.
[PubMed Abstract](#) | [Publisher Full Text](#)
- von Wintersdorff CJH, Penders J, van Niekerk JM, et al.: **Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer.** *Front Microbiol.* 2016; **7**: 173.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Forsberg KJ, Reyes A, Wang B, et al.: **The shared antibiotic resistome of soil bacteria and human pathogens.** *Science.* 2012; **337**(6098): 1107–11.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Karkman A, Do TT, Walsh F, et al.: **Antibiotic-Resistance Genes in Waste Water.** *Trends Microbiol.* 2018; **26**(3): 220–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Che Y, Xia Y, Liu L, et al.: **Mobile antibiotic resistome in wastewater treatment plants revealed by Nanopore metagenomic sequencing.** *Microbiome.* 2019; **7**(1): 44.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Thanner S, Drissner D, Walsh F: **Antimicrobial Resistance in Agriculture.**

- mBio*. 2016; 7(2): e02227–15.
[Publisher Full Text](#)
11. San Millan A: **Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context.** *Trends Microbiol.* 2018; 26(12): 978–85.
[PubMed Abstract](#) | [Publisher Full Text](#)
 12. Huddleston JR: **Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes.** *Infect Drug Resist.* 2014; 7: 167–76.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 13. McInnes RS, McCallum GE, Lamberte LE, *et al.*: **Horizontal transfer of antibiotic resistance genes in the human gut microbiome.** *Curr Opin Microbiol.* 2020; 53: 35–43.
[PubMed Abstract](#) | [Publisher Full Text](#)
 14. Johnston C, Martin B, Fichant G, *et al.*: **Bacterial transformation: distribution, shared mechanisms and divergent control.** *Nat Rev Microbiol.* 2014; 12(3): 181–96.
[PubMed Abstract](#) | [Publisher Full Text](#)
 15. Chen I, Dubnau D: **DNA uptake during bacterial transformation.** *Nat Rev Microbiol.* 2004; 2(3): 241–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
 16. Jonas DA, Elmadfa I, Engel KH, *et al.*: **Safety considerations of DNA in food.** *Ann Nutr Metab.* 2001; 45(6): 235–54.
[PubMed Abstract](#) | [Publisher Full Text](#)
 17. Hasegawa H, Suzuki E, Maeda S: **Horizontal Plasmid Transfer by Transformation in *Escherichia coli*: Environmental Factors and Possible Mechanisms.** *Front Microbiol.* 2018; 9: 2365.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 18. Sun D: **Pull in and Push Out: Mechanisms of Horizontal Gene Transfer in Bacteria.** *Front Microbiol.* 2018; 9: 2154.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 19. Stalder T, Rogers LM, Renfrow C, *et al.*: **Emerging patterns of plasmid-host coevolution that stabilize antibiotic resistance.** *Sci Rep.* 2017; 7(1): 4853.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 20. San Millan A: **Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context.** *Trends Microbiol.* 2018; 26(12): 978–85.
[PubMed Abstract](#) | [Publisher Full Text](#)
 21. Li X, Wang Y, Brown CJ, *et al.*: **Diversification of broad host range plasmids correlates with the presence of antibiotic resistance genes.** *FEMS Microbiol Ecol.* 2016; 92(1): fiv151.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 22. McGann P, Snesrud E, Maybank R, *et al.*: ***Escherichia coli* Harboring *mcr-1* and *bla*_{CTX-M} on a Novel IncF Plasmid: First Report of *mcr-1* in the United States.** *Antimicrob Agents Chemother.* 2016; 60(7): 4420–1.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 23. Liu YY, Wang Y, Walsh TR, *et al.*: **Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study.** *Lancet Infect Dis.* 2016; 16(2): 161–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
 24. Kruse H, Sørum H: **Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments.** *Appl Environ Microbiol.* 1994; 60(11): 4015–21.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 25. Chung CT, Niemela SL, Miller RH: **One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution.** *Proc Natl Acad Sci U S A.* 1989; 86(7): 2172–5.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 26. Top EM, Moenne-Loccoz Y, Pembroke T, *et al.*: **Phenotypic Traits Conferred by Plasmids.** In: Thomas CM, editor. *Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. eBook. London: CRC Press; 2014.
 27. van Elsland JD, Fry J, Hirsch PR, *et al.*: **Ecology of Plasmid Transfer and Spread.** In: Thomas CM, editor. *Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. eBook. London: CRC Press; 2014.
 28. Bale MJ, Day MJ, Fry JC: **Novel method for studying plasmid transfer in undisturbed river epilithon.** *Appl Environ Microbiol.* 1988; 54(11): 2756–8.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 29. Hill KE, Weightman AJ, Fry JC: **Isolation and screening of plasmids from the epilithon which mobilize recombinant plasmid pD10.** *Appl Environ Microbiol.* 1992; 58(4): 1292–300.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 30. Smalla K, Haines AS, Jones K, *et al.*: **Increased abundance of IncP-1beta plasmids and mercury resistance genes in mercury-polluted river sediments: first discovery of IncP-1beta plasmids with a complex *mer* transposon as the sole accessory element.** *Appl Environ Microbiol.* 2006; 72(11): 7253–9.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 31. Kav AB, Sasson G, Jami E, *et al.*: **Insights into the bovine rumen plasmidome.** *Proc Natl Acad Sci U S A.* 2012; 109(14): 5452–5457.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 32. Kothari A, Wu YW, Chandonia JM, *et al.*: **Large Circular Plasmids from Groundwater Plasmidomes Span Multiple Incompatibility Groups and Are Enriched in Multimetal Resistance Genes.** *mBio.* 2019; 10(1): e02899–18.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 33. Templeton AR: **The complexity of the genotype-phenotype relationship and the limitations of using genetic “markers” at the individual level.** *Sci Context.* 1998; 11(3–4): 373–89.
[PubMed Abstract](#) | [Publisher Full Text](#)
 34. Clark AG: **Limits to Prediction of Phenotypes from Knowledge of Genotypes.** In: Clegg M. T., Hecht M. K., J. MR, editors. *Evolutionary Biology*. Boston, MA: Springer; 2000.
[Publisher Full Text](#)
 35. Fang Z, Zhou H: **Identification of the conjugative and mobilizable plasmid fragments in the plasmidome using sequence signatures.** *Microb Genom.* 2020; 6(11): mgen000459.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 36. Ricker N, Spoja BS, May N, *et al.*: **Incorporating the plasmidome into antibiotic resistance surveillance in animal agriculture.** *Plasmid.* 2021; 113: 102529.
[PubMed Abstract](#) | [Publisher Full Text](#)
 37. WHO: **Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2016-2017.** Geneva: World Health Organization, 2017.
[Reference Source](#)
 38. Guo XP, Yang Y, Lu DP, *et al.*: **Biofilms as a sink for antibiotic resistance genes (ARGs) in the Yangtze Estuary.** *Water Res.* 2018; 129: 277–86.
[PubMed Abstract](#) | [Publisher Full Text](#)
 39. Sharma D, Misba L, Khan AU: **Antibiotics versus biofilm: an emerging battleground in microbial communities.** *Antimicrob Resist Infect Control.* 2019; 8: 76.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 40. Stalder T, Cornwell B, Lacroix J, *et al.*: **Evolving Populations in Biofilms Contain More Persistent Plasmids.** *Mol Biol Evol.* 2020; 37(6): 1563–76.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 41. Winkler ML, Papp-Wallace KM, Hujer AM, *et al.*: **Unexpected challenges in treating multidrug-resistant Gram-negative bacteria: resistance to ceftazidime-avibactam in archived isolates of *Pseudomonas aeruginosa*.** *Antimicrob Agents Chemother.* 2015; 59(2): 1020–9.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 42. Sanz-García F, Hernando-Amado S, Martínez JL: **Mutation-Driven Evolution of *Pseudomonas aeruginosa* in the Presence of either Ceftazidime or Ceftazidime-Avibactam.** *Antimicrob Agents Chemother.* 2018; 62(10): e01379–18.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 43. Duedu KO: **“Replication Data for PAMRS Workflow: A rapid screening workflow for phenotypic characterization of antibiotic resistance in plasmidomes”.** Harvard Dataverse, V1, UNF:6:cRh/M1HAuXnMjdN4vovBw== [fileUNF], 2021.
<http://www.doi.org/10.7910/DVN/0QZJQZ>

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Duedu *et al.* propose a novel workflow to screen for phenotypic antimicrobial resistance (AMR) contained within plasmids. Plasmids present one of the greatest threats to AMR spread because they can transmit between bacteria, including among the different hosts they are colonizing. A strength of the PAMRS workflow is that it was validated using plasmids isolated from chickens, cattle, and humans. Although the main objective was to report on the methods, an interesting finding was the disproportionate resistance found in chickens raised in poultry farms and the greater diversity of AMR expressed in humans compared with chickens or cows.

Major comments:

- The methods are slightly unclear:
 - Why did some plates have antibiotics incorporated into the agar while others used antibiotic discs? (It was mentioned in the results but would be better explained in the methods.) It would also be good to describe where you picked colonies from when using antibiotic discs since there is a limited zone of diffusion.
 - How were clones generated by the above methods compared?
 - What is the rationale for different ratios of plasmids?
 - There are several references to the E. coli being transformed. How many transformation events were there? It would be best to present the methods sequentially.
- It is unclear to me why chickens had to be sacrificed for obtaining fecal samples. It would be helpful to clarify why it was necessary.
- "Overall, it was evident that at least one plasmidome contained a plasmid that confers resistance to each of the thirteen antibiotics investigated." This is somewhat misleading. No single source contained resistant phenotypes against all 13 antibiotics.
- "With the very high levels of resistance from the cattle and human samples, there is the

need to review the use of the drug in these populations as well as other areas.” - It would be helpful to cite the agricultural practices around antibiotic use in this area to know whether that is actually contributing. Also, it is difficult to infer the high levels of resistance because everything is presented piecemeal in the text. Figure 6A would be clearer as a table that includes both numbers and percentage of resistance conferred by plasmids. This could replace some of the text, which is hard to follow.

- What is the significance of figure 6B? Does it suggest that certain resistance phenotypes cluster on plasmids? It would be helpful to explain.
- “Another key finding from this study is that humans compared to chickens and cattle appear to harbour more antibiotic resistant plasmids.” Since there were only 19 non-representative human samples, this conclusion seems like a reach.
- The manuscript would be strengthened by elaborating on the context and relevance. For example, are the observed rates of resistance comparable to other studies in Ghana or elsewhere? See Yevutsey *et al.* and Newman *et al.*

Minor comments:

- Figure 4 should accompany the methods, not the results.
- It would be good to provide some background on what determines phenotypic expression of plasmid-mediated AMR genes. For example, see Hulton *et al.* (1990).
- “It has been demonstrated that, mutation-driven evolution of resistance to ceftazidime in *Pseudomonas aeruginosa* could develop in a span of just 30 days of exposure to increasing concentrations of the drug.” - Is this mediated by plasmids? Otherwise, I’m not sure what its relevance is.
- It sounds like you are stating your objective in the discussion. I recommend putting this in the introduction.
- The manuscript would benefit from additional English grammar editing.

Other comments:

- It would be interesting to validate these findings with genetic approaches to see which AMR genes are present and expressed between hosts. Perhaps this could be a consideration for future research. For example, does ceftazidime/ceftriaxone phenotypic resistance represent an ESBL genotype?

Is the rationale for developing the new method (or application) clearly explained?

Partly

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: AMR epidemiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.