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Wastewater surveillance of antimicrobial resistance in human populations: a systematic review

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Abstract

Wastewater-based surveillance of antimicrobial resistance (AMR) may facilitate convenient monitoring of population-level AMR prevalence without the healthcare-associated bias and data collection restrictions inherent to clinically oriented systems. However, differences in study design and methodology likely contribute to differences in outcomes and interpretation, limiting reproducibility, reliability and meta-analysis. We therefore systematically reviewed studies using wastewater for AMR surveillance in human populations to identify optimal practices to detect wastewater-human AMR correlations.

We evaluated 7,063 records and 174 full-text methods in a two-stage screen; 20 studies were included. Risk of bias assessment divided studies into high-risk (n=3), low-risk (n=3) and unclear-risk (n=14). Most studies detected wastewater-human AMR correlations (n=15) but only six studies identified statistically significant associations, most via culture-independent approaches (n=5). Genomic approaches also facilitated higher-resolution AMR monitoring whereas culture-based studies primarily undertook observational comparisons of specific organisms and phenotypic AMR profiles. Studies identifying wastewater-human AMR correlations were consistently associated with sampling wastewater influent irrespective of other methodological approaches. For longitudinal studies, a timeframe of >=6 months was similarly associated. Most influent studies identifying wastewater-human AMR correlations used composite (n=5) or flow-proportional wastewater sampling methods (n=4); however, grab sampling was commonest overall (n=6) and generally appeared similarly effective.

Wastewater-based surveillance of AMR in human populations appears relatively robust, with most included studies reporting a correlation despite high diversity in study design and

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methodology. Our review supports sampling of wastewater influent using composite sampling (at a minimum) as a standard. Impacts of other methodological approaches are less clear; however, a minimum timeframe of six months for longitudinal studies, and increased sampling coverage for culture-independent studies to enable adequate biostatistical analyses appear sensible. As this relatively new field grows, more studies with clear wastewater-based population-level AMR surveillance aims are needed to better determine the impact of confounding features and validate comprehensive “best practice” protocols.

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Introduction

Antimicrobial resistance (AMR) is a significant threat to global health[1] and the treatment of infectious diseases, and is compounded by diverse drivers[2,3] facilitating emergence and spread. AMR surveillance is therefore critical to understanding trends and monitoring interventions, as prioritised in the World Health Organisation's global AMR action plan[4]. Large networks dedicated to sharing AMR data have been established to meet this need, including the European Antimicrobial Resistance Surveillance Network (EARS-Net) and the Global Antimicrobial Resistance Surveillance System (GLASS). However, current surveillance methods are limited by the reliance on patient-level sampling, which is often affected by selection bias towards healthcare-associated settings[5]; this does not reliably capture AMR prevalence in commensal organisms and in the community, thought to silently constitute most of the true AMR burden[6–8]. Additionally, reliance on routine clinical microbiology results often restricts data collection to a limited subset of culturable species, and focuses predominantly on susceptibility phenotypes with limited genotyping. This lack of genotyping hampers the surveillance of high-risk AMR-associated clones and the horizontal transfer of AMR determinants[9].

Wastewater-based AMR surveillance has the potential to avoid biases in current surveillance methods by simultaneously sampling both healthcare- and community-associated populations[10]. The approach has already been successful in illicit drug monitoring[11] and pathogen (particularly enterovirus) surveillance[12,13] but its application to AMR surveillance is relatively new[7]. Recent wastewater AMR studies have investigated seasonal/geographic AMR distributions[14], quantified global abundances of AMR genes[6] and identified

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correlations between wastewater and clinical AMR surveillance data[15]. However, differences in methodology and study design among wastewater AMR studies likely contribute to differences in outcomes/interpretations. For example, the relative impact of wastewater sampling approaches on study outcomes are not well understood, including the effects of grab sampling (i.e. taking single samples at one timepoint) in collecting homogenous solids/unrepresentative samples[16], or sampling in the presence of unrepresentative, contaminating AMR-associated point sources. Difficulties in standardising AMR testing across traditional surveillance networks[9] could be, for example, circumvented by using metagenomic sequencing to universally probe wastewater resistomes[17,18]. However, despite increasing research in this area, there has not been an attempt to review the data available and assess the remaining knowledge gaps.

We systematically reviewed studies using wastewater for AMR surveillance in human populations, seeking to identify practices that could optimise identification of the correlation of wastewater-human AMR for surveillance purposes. This review examines both study design and methodology to identify the relative potential impact of these factors on study outcomes, and to highlight any limitations and recommendations for future research.

Materials and Methods

For this systematic review, we adopted the “Population Intervention Comparator Outcome” (PICO) framework using the following domains: Wastewater, antimicrobial resistance, bacteria and public health surveillance/methods. The search string was developed through

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iterative preliminary searches in consultation with a librarian experienced with systematic reviews; subject headings/operators were adapted for each bibliographic database (full search strings are presented in **Supplementary dataset 1**). Searches were conducted on 01/02/2019 in: Ovid MEDLINE (1946-present), Ovid EMBASE (1974-present), Ovid Global Health (1973-present), Ovid CAB Abstracts (1973-present), Scopus and Web of Science Core Collection. Searches were updated on 21/10/2019 using identical search strings. Search results were limited to the English language and de-duplicated.

Study titles/abstracts were screened (**Fig.S1**) to determine if the study: (i) was primary research, (ii) collected human-associated wastewater, (iii) reported AMR prevalence, and (iv) compared a wastewater dataset against another non-wastewater dataset of relevance. Studies performing self-defined wastewater-based surveillance of AMR (i.e. studies explicitly using wastewater for population-level AMR surveillance) were purposely included using parallel criteria. If it was unclear whether a study met all criteria based on title and abstract alone, the study was passed onto the next stage. For studies passing the initial screen, full-text methods were reviewed and studies were included if they: (i) analysed wastewater samples from a wastewater treatment works (WwTW) and represented a human population in their non-wastewater dataset, or (ii) conducted a self-defined wastewater-based AMR surveillance study as part of the study aim.

All studies were screened by a single reviewer (KKC); two other reviewers (LB, NSi) independently re-screened a random 10% subset of retrieved records to estimate Cohen's Kappa score[19] as a measure of inter-reviewer reliability beyond chance. A score above 0.75

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was interpreted as representing excellent agreement beyond chance[20,21]. Consensus was reached by discussion in the case of disagreements.

For included records, data were extracted by a single reviewer (KKC) and checked by a second (LB) using a pre-tested data extraction form piloted on five random included records (**Supplementary dataset 2**), including: general characteristics (publication year, geographic location), study design (study aim, sampling strategy, sampling point, sampling methods, AMR detection methods, human component, sample sizes) and outcomes (results of wastewater-human comparison with respect to AMR). Statistical methods or modelling approaches were also recorded if used, and a summary of relevant findings and additional points of interest as judged by the reviewer were documented.

Included studies underwent a risk of bias assessment by two independent reviewers (KKC, LB). Risk of bias was assessed using a qualitative, modified approach based on the Cochrane risk of bias tool[22] addressing five bias domains (see below); this focused on systematic methodological differences as reported outcomes were highly diverse. Selection bias was defined as differences introduced across sampled sites using different sampling methods. Performance bias was defined as differences introduced across comparison groups using different AMR detection approaches. Attrition bias was defined as differences introduced by incomplete outcome data (e.g. incomplete data entry across comparison groups or longitudinally). Reporting bias was defined where outcomes may have been measured but not reported/disproportionately reported. Lastly, other bias was defined as confounding due to the likely presence of AMR/AMR-influencing sources which were not considered and

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may have affected outcomes. Studies at "high-risk" of bias were those with inconsistencies in sampling, AMR detection methods, measurement and reporting of outcomes; "low-risk" studies broadly maintained consistency across comparators. If information present was insufficient to assess risk of bias, the classification "unclear" was assigned. Discrepancies were resolved by discussion, and an overall qualitative measure (high, low, unclear) was assigned to each study.

The complete review protocol is available on PROSPERO at: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019134946; (registered: 14/05/2019). A PRISMA checklist is included in the Supplement (**Supplementary dataset 3**).

Results

Summary of included studies

7,063 de-duplicated records were retrieved, 174 full-text methods were reviewed, and 20 studies were included (**Fig.1**). Inter-rater reliability (on n=701 studies) was supported (Cohen kappa score=0.76)[20,21]; screening conflicts were observed for 27 studies. 9/20 studies were self-defined wastewater AMR surveillance studies; 11 studies performed wastewater AMR surveillance indirectly by investigating: AMR transmission into wastewater environments (n=6); AMR transmission routes to humans (n=3); and epidemiological links between wastewater and human bacterial isolates (n=2). Study summaries are presented in **Tables 1** (aims, overview and overall risk of bias) and **2** (methodology).

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Three studies were judged at high-risk of bias, three at low-risk and 14 had an unclear-risk (**Table 1**, **Supplementary dataset 4**). Selection bias was present in six studies[6,23–27] where 5/6 sampled multiple wastewater sources using different sampling methods. Performance bias was not present in any studies. Attrition bias occurred in two studies as missing timepoints due to logistical factors (WwTW closure/sampling permissions[15], missing datapoints[28]). Reporting bias was present in three studies where sample sizes or antibiotic phenotyping data were incompletely reported for ≥1 group[24,29,30]. Confounding bias occurred in four studies[27,31–33] which did not report the presence/absence of sewer inputs. Studies assigned an unclear risk of bias did not provide sufficient information to evaluate bias, such as partial description of sewer inputs or absent descriptions of sampling methods.

Amongst included studies, samples were obtained from 67 countries, although most (48/67) were represented as part of a single global study[6] (**Fig.2**). Most studies (n=13) sampled Europe and Central Asia, and sampling was skewed towards high-income countries (high income [n=14], middle income [n=8], low income [n=1]) (**Fig.S2**). Two studies covered multiple regions and income classifications. Publication dates ranged from 2007-2019, with just over half published in the last three years (n=11). Sampling years ranged from 2004-2017, the most common being 2011-2016 (n=11); only one study[27] had actually sampled within the last three years.

Wastewater sampling point and method

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WwTW influent was the most common single sampling point (n=7), followed by activated sludge (n=2), WwTW effluent (n=1) and untreated drainage ditch waste (n=1). Remaining studies sampled WwTW influent and at least one other wastewater source (n=6, [effluent =4], [effluent and refined=1], [pre-influent=1]), or the sampling point was unreported (n=3). The most common collection method was grab sampling (n=7), followed by composite sampling (n=5), flow-proportional sampling (n=4) and mixed composite/flow-proportional sampling (n=1). Three studies did not report collection methodology.

Amongst 15 studies observing a wastewater-human AMR correlation, the most common sampling point was WwTW influent (n=10; conducted via composite [n=5], flow-proportional [n=3], grab [n=1], mixed composite/flow-proportional [n=1] sampling methods). Three studies observed no relationship between wastewater and human AMR, sampling either influent via flow-proportional sampling, effluent with unreported methods, or drainage ditches via grab sampling. The remaining two studies were inconclusive and sampled influent and/or effluent with unreported methods (Fig.3A).

Longitudinal sampling and study timeframes

Thirteen studies conducted longitudinal sampling with total sampling timeframes ranging from 3-14 months (median: 12 months, IQR: 6-12). 10/13 studies reported sampling intervals, ranging from two weeks to six months (median: one month, IQR: 1-1.5). The remaining studies did not conduct longitudinal sampling (n=6) or were unclear in their approach (n=1).

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Eight longitudinal studies identified wastewater-human AMR correlations, with the median timeframe and sampling interval range being 12 and 0.5-6 months respectively. The remaining five reported no correlation or were inconclusive; the median timeframe and sampling interval range in these studies were 11 and 0.5-2 months respectively. All snapshot studies and one study with unclear timeframe also identified wastewater-human AMR correlations (Fig.3B).

Nine studies performed direct wastewater-human AMR comparisons where the human population sampled directly contributed to the WwTW sampled. Six studies performed indirect comparisons where the human population sampled did not contribute to the WwTW sampled. One study undertook both evaluations, and four were unclear in this respect. For direct studies, 5/9 reported wastewater-human AMR correlations; however, all six indirect studies reported wastewater-human AMR correlations.

Wastewater treatment works and sewershed

The median number of WwTW sampled was three (IQR: 1-6), with a median of 33 samples taken (IQR: 20-67; Fig.S3). A wide range of WwTW sizes was present among studies reporting this feature (11/20), with a median population equivalent (PE) of 91,577 (IQR: 16,462-192,450). Seven studies additionally reported mean flow entering WwTWs per day (m^3/d) (median: 25,617.6, IQR: 6,780.8-57,844.8) (**Fig.S4**). With respect to sewer inputs, 11/20 studies at least partly defined the presence/absence of distinct wastewater types

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entering the sampled WwTW. These included: hospital effluent (n=9), agricultural waste (n=2), industry effluent (n=2), abattoirs (n=1), nursing homes (n=1) and tourist facilities (n=1). The median number of WwTWs sampled (19/20 reporting) was three (IQR: 5, range: 0-53; one self-defined AMR surveillance study sampled simpler wastewater irrigation instead of a WwTW).

Increasing the number of WwTWs sampled appeared to be a feature of studies identifying a wastewater-human AMR correlation; WwTW PE/flow was also more commonly reported in these studies (**Fig.S3-4)**. With respect to sewer inputs, 7/15 studies identifying a wastewater-human AMR correlation reported WwTWs received at least one distinct wastewater type (e.g. hospital or animal effluent), 1/15 with none and the remaining studies did not report this feature. Studies that did not identify any wastewater-human AMR correlation or those that were inconclusive either did not report inputs (n=3) or reported at least one potential input (n=2).

Evaluation of AMR: culture, quantitative PCR (qPCR) and sequencing

AMR test approaches across all studies consisted of three main categories: predominately culture-based (n=11), direct qPCR-based (n=2) and DNA sequencing-based (n=7).

Culture-based approach (n=11)

Eleven studies used culture-dependent antimicrobial susceptibility testing (AST) methods to evaluate cultured isolates, namely: disk diffusion-only (n=3), disk diffusion and E-tests (n=2),

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disk diffusion and microbroth dilution (n=2) and microbroth dilution-only (n=4). Eight culture-dependent studies also used targeted genotyping methods to detect gene markers and relatedness of isolates, namely: microarray (n=1); PCR (n=3); PCR and pulse field gel electrophoresis (PFGE) (n=3); and qPCR (n=1). All culture-based studies investigated specific target species, including: *Enterococcus faecium* (n=2); *Enterococcus* spp. (n=1); *Escherichia. coli* (n=5); *Salmonella* spp. and *E. coli* (n=1); Enterobacteriaceae, *S. aureus* and *Enterococcus* spp. (n=1); and *Staphylococcus. aureus* (n=1). Specific resistance mechanisms and/or phenotypes were also investigated in eight studies: ampicillin (n=1); carbapenem, methicillin and vancomycin (n=1); gentamicin (n=2); methicillin (n=1); third generation cephalosporins (n=1); and vancomycin (n=2). The remaining three studies instead reported broad resistance profiles for 10-15 antibiotics. Of the eight studies which also performed targeted genotyping, studies targeted extended-spectrum beta-lactamase (ESBL) & carbapenemase (n=2) genes, ESBL genes-only (n=1), carbapenemase genes-only (n=1), aminoglycoside resistance-conferring genes (n=2), glycopeptide resistance-conferring genes (n=1) and *mec*A (n=1) (**Table 1, Fig.S5**). Distributions of target organism/phenotypes/number of target genes are visualised against outcome and AMR evaluation in (**Fig.S5**).

Most culture-based studies did not perform any statistical analyses (10/11) with the exception of one study[34] which modelled the relationship between resistance rates (defined in the study as the proportion of resistant isolates) in wastewater (WwTW influent and hospital effluent) and clinical isolates (hospital and primary care). For this study, a significant correlation was found between mean resistance rates in: (i) hospital wastewater and hospital clinical isolates, and (ii) WwTW influent and primary care urine isolates. This study

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also observed mean resistance rates were lower in influent compared to urine isolates (twofold difference in five antibiotics) whereas similar rates were found between hospital wastewater and hospital clinical isolates. An exception was cefadroxil resistance which was more prevalent in influent than in primary care isolates. Despite the cefadroxil difference, rates of ESBL-positive isolates were significantly higher in clinical isolates across settings.

Observational wastewater-human AMR relationships were reported for eight studies comparing wastewater isolates to: clinical isolates[16,26,29,31,35,36] (n=6), national AMR data[24] (n=1) and faecal isolates from healthy carriers[27] (n=1). Among clinical isolate studies, the most common observation was the sharing of drug resistance to specific antimicrobial classes[16,26], multi-drug resistance[35], specific resistance profiles[26,36], ESBL genes[36] and drug-resistant species[29], between wastewater and clinical isolates. Two clinical isolate-based studies also observed a correlation between wastewater isolates cultured and study country outbreaks and/or endemic circulation of resistant clones[26,31]. The study investigating faecal isolates from healthy carriers[27] reported correlation of the top four resistance phenotypes (ciprofloxacin, tetracycline, erythromycin, fosfomycin) between wastewater and faecal isolates, but with higher prevalence of resistance to these four antibiotics, multi-drug resistance and vancomycin-resistant enterococci in wastewater. Lastly, a comparison with national resistance data showed increasing resistance in wastewater isolates reflected increases in resistance reported for clinical blood isolates[24].

The remaining two culture-based studies reported no wastewater-human correlation, or an inconclusive outcome. Both these studies investigated gentamicin resistance but in different

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species (*Enterococcus* spp. and *E. coli*). The study[32] focussed on *Enterococcus spp.* found high clonal diversity and low genetic relatedness amongst cultured strains using PFGE, contrasting with the observation that AMR phenotype at the strain level matched national levels. The *E. coli*-focussed study[23] found no evidence of strain similarity using PFGE and observed no association in gentamicin resistance gene prevalence between clinical and wastewater isolates.

Direct qPCR-based approach (n=2)

Two studies directly detected AMR genes using qPCR of 229 and eight resistance genes respectively. Only the study[15] targeting 229 resistance genes performed statistical testing but both reported a relationship between wastewater AMR and national AMR data.

The study targeting 229 resistance genes compared a longitudinal survey of 13 European WwTWs across seven countries with contemporaneous surveillance data from EARS-Net. The relative abundance of influent AMR genes clustered significantly based on high versus low national antibiotic consumption, with AMR gene distributions mirroring EARS-Net-described north-to-south and west-to-east geographic gradients. A higher relative abundance of most AMR gene classes was observed in high antibiotic consumption countries except for tetracycline and macrolide-lincosamide-streptogramin B resistance genes. The study also performed counts of antibiotic-resistant culturable bacteria from samples but showed no significant correlation between counts and AMR gene quantification.

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The second qPCR-based study reported relationships between wastewater samples in Tunisia and Spain, and respective national AMR surveillance data. Intra-country comparisons showed the most commonly recovered beta-lactamase gene (bla_{TEM}) and quinolone resistance genes *qnr*S and *qnr*A in wastewater were also most commonly reported in clinical surveillance. This was not however true for CTX-M-9 group ESBL genes, where a higher wastewater prevalence was observed. Inter-country comparisons highlighted higher AMR gene detection in samples from Spain coinciding with higher antibiotic use, and high *mec*A prevalence thought to be potentially consistent with high-pig farming densities.

DNA sequencing-based approach (n=7)

Seven studies sequenced DNA for AMR detection, including: metagenomic sequencing-only (n=2), metagenomic and functional genomic sequencing (n=1), and whole genome sequencing (WGS) of cultured isolates (n=4). The single isolate WGS studies targeted *E. faecium* (n=1), *E. coli* (n=2) or *Pseudomonas aeruginosa* (n=1). The WGS studies also investigated specific genetic determinants conferring resistance to carbapenems (n=1), third generation cephalosporins (n=2) and vancomycin (n=1).

Biostatistical evaluations of sequencing data were performed in all (6/7) but one study[37]. Four studies reported a significant relationship between wastewater and human AMR using WGS (n=3) and metagenomics (n=1). Of these, two WGS studies compared wastewater isolates (*E. coli* and *E. faecium*) with same-species bacteraemia isolates using core genome

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phylogenies. In the *E. faecium* study[38], wastewater and clinical isolates were phylogenetically interspersed, indicating mixing, with divergence events implying recent local emergence and dissemination. Each WwTW sampled also contained genetically diverse populations but these remained comparable to diversity in national bloodstream infection isolates. Network analysis showed geographic clustering of WwTWs and bloodstream isolates, and linked bloodstream isolates to 9/20 WwTWs (three without hospital sewer input). The *E. coli* study[39] found the top three most clinically common ST types and ESBL gene ($bla_{CTX\text{-}M\text{-}15}$) were also found in all but one WwTW. Prevalence of common $bla_{CTX\text{-}M}$ genes in wastewater and clinical isolates also substantially overlapped ($bla_{CTX\text{-}M\text{-}15}$: 64% vs. 49%; $bla_{CTX\text{-}M\text{-}14}$: 10% vs. 6%; $bla_{CTX\text{-}M\text{-}27}$: 10% vs. 10%) as well as sharing of 56/70 other AMR gene variants. Carbapenem and colistin resistance were not detected, consistent with low local resistance prevalence to these antibiotics.

Another WGS study[28] reporting a wastewater-human AMR association compared wastewater with community-acquired UTI ESBL-*E. coli* isolates from residents in the WwTW catchment, identifying significantly higher single- and multi-drug resistance in urine isolates compared to those from wastewater even when adjusting for the clinically common ST131. Both community and wastewater samples shared $bla_{CTX\text{-}M\text{-}15}$ as the most commonly identified ESBL gene and shared similar prevalence of other *bla* genes ($bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1B}$) except for $bla_{CTX\text{-}M\text{-}14}$ which was more prevalent in wastewater.

The final study[6] reporting a wastewater-human AMR correlation conducted a large-scale cross-sectional global wastewater survey and compared resistomes to multiple

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epidemiological variables. This study showed total AMR gene abundances from a given site/country correlated with sanitation and general health metrics, with up to 89% of observed resistome variation significantly explained regardless of within-sample AMR gene diversity. A high human development index (i.e. generally high-income countries) was linked to significantly lower abundance of AMR genes in wastewater metagenomes.

The remaining 3/7 sequencing-based studies reported no relationship[33] between wastewater and human AMR or inconclusive results[33,40]. One inconclusive study conducted a metagenomics-based longitudinal surveillance study[40] comparing resistomes generated from drainage ditch wastewater and contemporaneous local surveillance data consisting of household morbidity and healthcare usage, including diarrhoea/fever cases and clinical faecal culture. Significant increases/decreases in resistome read abundance were not associated with increases in reported diarrhoea or clinical faecal isolates. However, non-significant increases in pathogen read abundances appeared to coincide with increased reported illness/clinic visits, possibly reflecting issues with methodological sensitivity (i.e. impact of sequencing depth) to detect significant changes.

The second inconclusive study was a metagenomic and functional metagenomic longitudinal evaluation of wastewater and human faecal samples collected from individuals within the WwTW catchment[33]. Characterisation of resistomes, including AMR gene network analysis, showed significantly higher phylogenetic species diversity and abundance of AMR proteins in wastewater (WwTW influent, street-access wastewater) compared to faecal samples. Faecal samples were significantly enriched for drug efflux mechanisms whereas wastewater

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samples contained more aminoglycoside acetyltransferase, class D β-lactamase, and dihydrofolate reductase genes. However, extensive sharing of sulphonamide-resistance conferring AMR genes was detected, indicating a possible resistance-specific relationship.

Lastly, a longitudinal study[37] reported low to no relationship between wastewater and human AMR based on its comparison of clinical and WwTW effluent carbapenem-resistant *P. aeruginosa* using PFGE, multilocus sequence typing (MLST), WGS to evaluate acquired carbapenemase genes, and culture-based AST. PFGE pulsotypes showed very limited overlap between clinical and wastewater samples (n=4). However, WGS showed dissemination of the bla_{VIM-2} carbapenemase gene in clinical and wastewater samples.

Interactions between study features

When assessing the distribution of study features relative to the study outcome, wastewater sampling point and AMR evaluation approach, studies identifying wastewater-human AMR correlations were consistently associated with sampling influent (**Fig.3**). All wastewater sampling methods also identified wastewater-human AMR correlations when influent was sampled, with composite sampling associated to studies identifying correlations regardless of AMR evaluation approach (**Fig.3A**). For longitudinal studies, a timeframe of >=6 months was most closely associated with the identification of a wastewater-human AMR correlation when sampling influent or sludge whilst using culture- or qPCR-based AMR evaluation (**Fig.3B**). The presence of sewer inputs with distinct wastewater types appeared to cluster with studies identifying a wastewater-human

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correlation where these sampled influent, irrespective of AMR evaluation approach (**Fig.3C**). No human dataset type was exclusively associated with a given outcome/sampling point. However, clinical isolate studies were common amongst those identifying wastewater-human AMR correlations, irrespective of sampling points (although most sampled influent or influent and effluent; **Fig.3D**). For additional plots see (**Figs.S6-8**).

Antibiotic prescribing and residue measurements

Five studies investigated the relationship between antibiotic prescribing/measured concentrations and AMR prevalence. National prescribing/usage was associated with qPCR-ARG abundances[30,41] and individual resistance phenotypes[32], but not metagenomic-ARG abundances[6]. Local clinical prescribing was associated with clinical resistances[36] while primary care prescribing showed no association with qPCR-ARGs. Antimicrobial residue measurements were not associated with either qPCR-[15] or metagenomic-ARG[6] abundances.

Discussion

In our review, most (15/20, 75%) studies reported that wastewater AMR reflected AMR in human catchment populations, but the extent to which this relationship could be evaluated was dependant on study design, sampling strategies, and genotypic versus phenotypic testing approaches; testing approach also determined outcome quality.

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As anticipated, we found WwTW influent is the most population-representative sampling point; previous studies have described transformation of microbial and AMR composition during treatment[42]. Sampling influent appears to be the single most important feature associated with identifying a human-wastewater AMR correlation (**Fig.3, Figs.S6-8**). However, several studies identifying such a correlation analysed treated wastewater, indicating transformed samples may remain useful for wastewater surveillance, potentially dependent on treatment process. However, only 3/8 non-influent studies reported WwTW treatment types, and evaluating the impact of these remains important as specific treatments may favour specific species/AMR determinants[43].

Composite and 24H flow-proportional sampling may be better than single grab sampling given that wastewater composition changes significantly over short timescales[44] and individual samples may be "flooded" by homogenous solid material[16]. However, several influent studies using grab-like composites with 2-3 sub-samples in quick succession, and non-influent studies using single grab samples, also demonstrated a human-wastewater AMR correlation. Grab sampling, which was the most commonly used method, is convenient and avoids significant autosampler-associated workload and capital costs. Despite potential sample homogeneity, half of all studies identifying a wastewater-human AMR correlation used grab samples in combination with culture-based/phenotypic approaches (**Fig.3A**). Grab samples may therefore be representative for such analyses although further research is needed to characterise the extent to which a single grab can accurately reflect temporal flux in AMR.

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Longitudinal studies demonstrating a human-wastewater AMR correlation typically included longer sampling intervals (six months versus two months for negative/inconclusive studies) and timeframes. For the highest resolution sampling campaigns[26,40] which used two week sampling intervals across different timeframes, one study observed an association between ampicillin-resistant wastewater and contemporaneous clinical isolates over 12 months, whereas the other found changes in metagenomic read abundances were not matched by changes in contemporaneous clinical surveillance over three months. Directly comparing these studies is difficult due to different methodologies, but the findings would support the fact that studies sampling over a timeframe of ≥6 months are more likely to capture associations with human population-level AMR (**Fig.3B**).

The unexpected finding that human-wastewater AMR correlations were not observed in some cases where the wastewater being sampled received direct inputs from the population being surveyed can likely be explained by study aims and methods. Most of these direct studies aimed to show fine-scale links in small catchment areas via biostatistical analyses whereas most indirect studies compared broad (often national) observations without statistical testing. Only 2/6 indirect studies reported significant relationships compared to 4/9 direct studies. Smaller catchments also limit sample sizes potentially obscuring signals from detection which may have contributed to unclear/negative outcomes[40]. When comparing direct studies, similar sampling methods and test approaches are seen, but amongst studies identifying wastewater-human AMR correlations, more WwTWs and higher numbers of wastewater samples were sampled per study (**Fig.S3**). This

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suggests higher sampling coverage may aid capturing fine-scale changes in smaller catchments.

The presence of specific sewer inputs from potentially major sources of AMR in wastewater (e.g. hospitals) was interestingly associated with positive outcomes (**Fig.3C**), and likely linked to whether the AMR mechanisms studied were hospital-associated/emerging or already widely disseminated in the community. For example, one study[39] sampled WwTWs with and without hospital input, and found the most clinically-prevalent *E. coli* ESBL gene was also present in all WwTWs – indicating widespread prevalence across settings. Another study[23] focussing on *E. coli* gentamicin resistance in hospital effluent, WwTW influent and domestic wastewater, found significantly lower prevalence in domestic wastewater than in both influent and hospital effluent – indicating likely nosocomial emergence. Only measuring hospital-associated wastewater in this context would therefore confound community AMR estimates. Importantly, half of all studies did not report sewer inputs at all, making interpretation of findings more difficult; a similar lack of reporting was observed for WwTW PE and flow estimates (**Fig.S4**).

Culture-dependent and independent test approaches were both associated with the identification of wastewater-human AMR correlations (culture-based studies: 9/11, culture-independent studies: 6/9). However, for the six studies that identified statistically significant positive correlations, most used culture-independent approaches (n=5). Culture-based approaches appear useful for reporting observational wastewater-human AMR correlations, but studies employing culture-independent methods are relatively species- and mechanism-

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agnostic, and may be more tractable and statistically robust for public health surveillance purposes. Combination approaches may capture the breadth and detail of genetic relationships between bacterial strains and important AMR genes.

For culture-based studies the evaluation of overlap between wastewater and human isolates is limited to comparisons of phenotypic AMR profiles, which may reflect significant differences in underlying genotypes, particularly for Gram-negative organisms such as Enterobacterales. Most studies[25,28,32,34,35] showed AMR prevalence in wastewater was lower, but common AMR phenotypes were frequently shared, indicating culture/phenotype-based surveillance of wastewater may be useful for low-resolution monitoring of AMR in clinical isolates. Targeted culture-based approaches could also pick up relevant high-risk drug-resistant clones (MRSA, VRE, CRE, MDR-*Salmonella* Typhimurium DT104)[26,27,29,35]. Increasing AMR prevalence in wastewater isolates appeared to mirror national clinical AMR rates in one study[24], although this study was subject to reporting bias.

Culture-independent approaches could be used for more detailed analyses such as exploratory ordination, phylogenetics, and statistical modelling. As a result, comparisons of relative AMR gene abundance, genomic relatedness and evolution, and resistome variation could be reported. In-depth genomic data may be more useful for higher-resolution AMR monitoring in clinical isolates and human carriage, but at a higher resource cost. In comparisons with prescribing data, targeted qPCR methods appeared to perform better than metagenomics which may reflect sensitivity trade-offs that occur with lower

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sequencing depth. However, neither approach correlated with wastewater antimicrobial concentrations; this may however be linked to detection limits and sample variability[6,15].

This review has some limitations. Our highly comprehensive search strategy meant that screening could not be carried out in duplicate; however, the risk of single reviewer bias was mitigated by validating the screening strategy with three reviewers on 10% of records. We excluded non-English publications, potentially missing some relevant studies. 11/20 included studies were not self-defined wastewater surveillance studies, and so the relevance of their findings to the systematic review question was based on reviewer judgement. Studies were highly diverse in reported design/outcomes, complicating standardisation for narrative synthesis. Finally, the premise of our review was based on the assumption that wastewater-human AMR correlations occur, and to identify study approaches which best characterise this phenomenon, but it may be that in some cases there is genuinely no association.

Conclusion

Overall, wastewater-based surveillance of population-level AMR appears relatively robust, with most included studies reporting wastewater-human AMR correlations despite high diversity in study design, methods and metadata. Our review suggests sampling of influent and composite sampling optimise the chance of identifying human-wastewater AMR correlations and are most suitable for wastewater-based AMR surveillance studies. Impacts of timeframe, comparison type and the number of WwTWs sampled were less clear based

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on the available data, however a minimum timeframe of six months appears sensible since longitudinal changes may be clearer over longer timescales. Biostatistical analyses likely benefit from increased sampling coverage of the catchment area by including more WwTWs and larger numbers of wastewater samples. A culture-independent approach facilitates agnostic detection and statistical synthesis but is more resource-intensive and may be limited in sensitivity by low sequencing depths; culture-based approaches however do enable low-resolution clinical surveillance and targeted detection of specific clones and/or AMR phenotypes. Studies should clearly report sewer inputs, WwTW features (e.g. PE, flow rates, treatment methods), sampling and sample processing methods, sampling intervals and timeframes. As this relatively new field grows, more studies with clear wastewater-based population-level AMR surveillance aims are needed to better determine individual feature contributions and confirm comprehensive “best practice” protocols.

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1 **FIGURES**

2 **Figure 1: Flowchart of studies identified, screened, and included in the systematic review.**

3

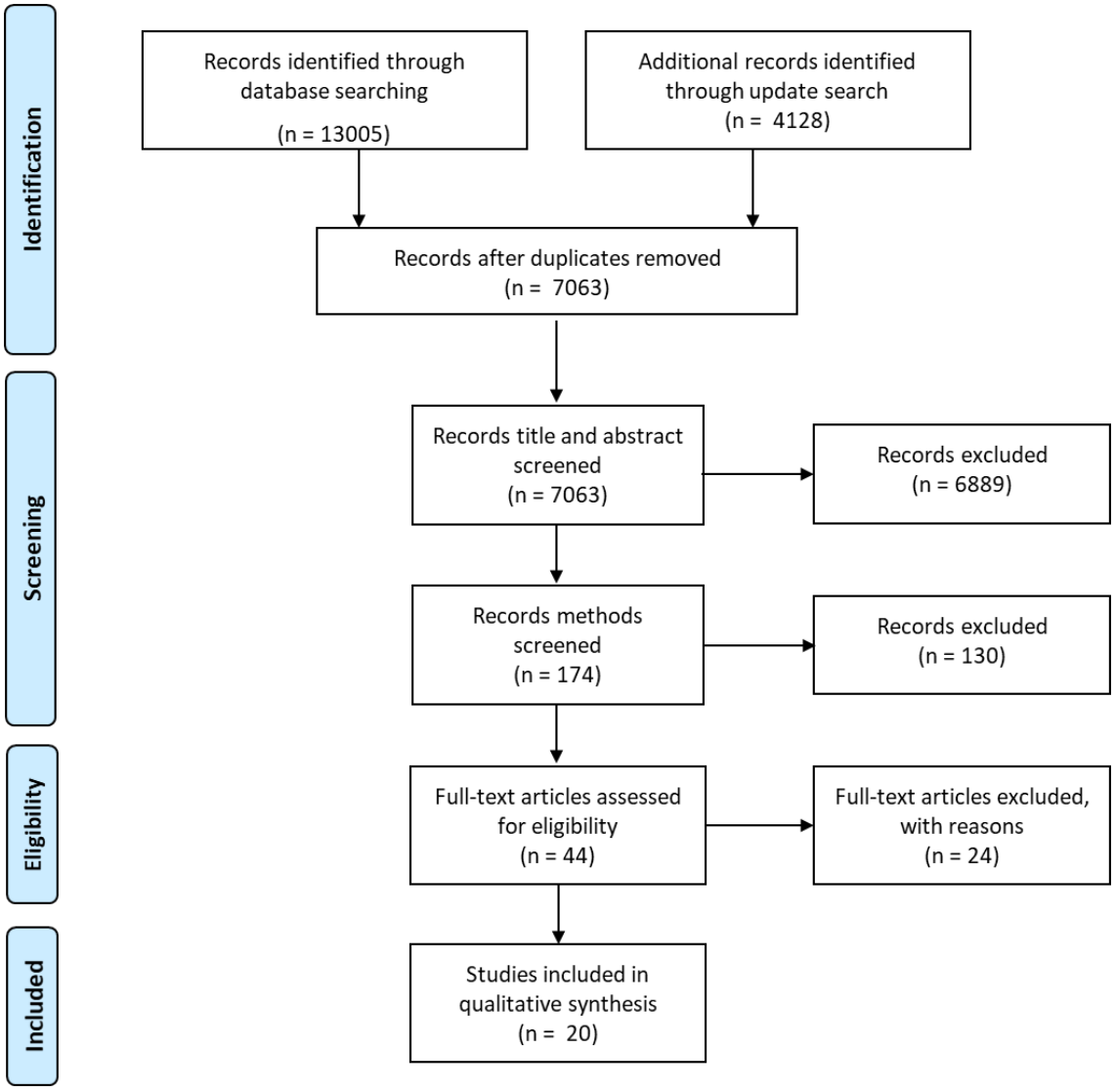

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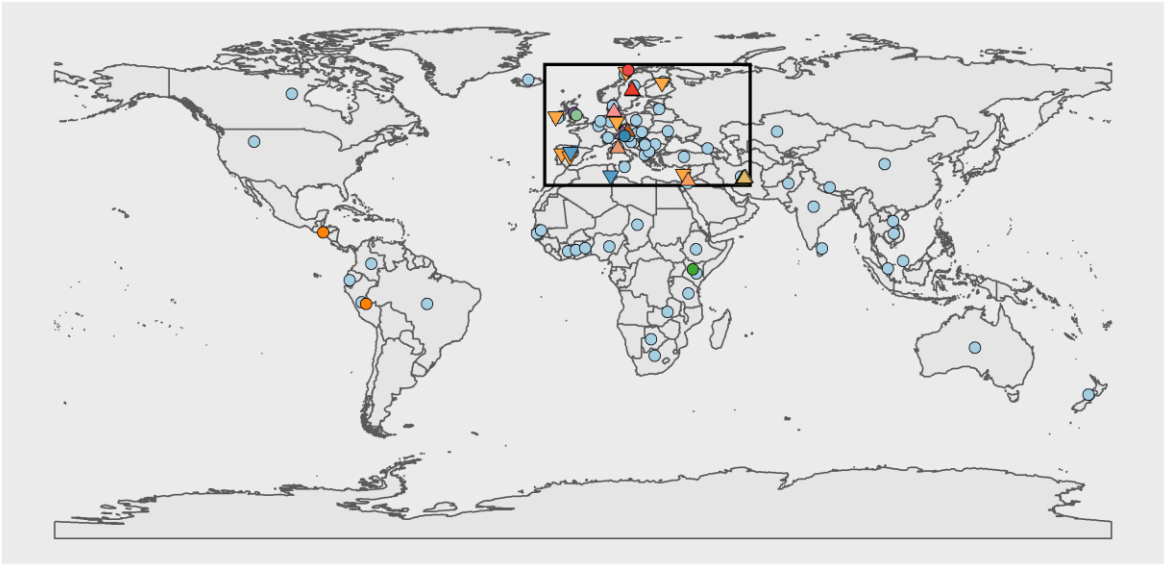
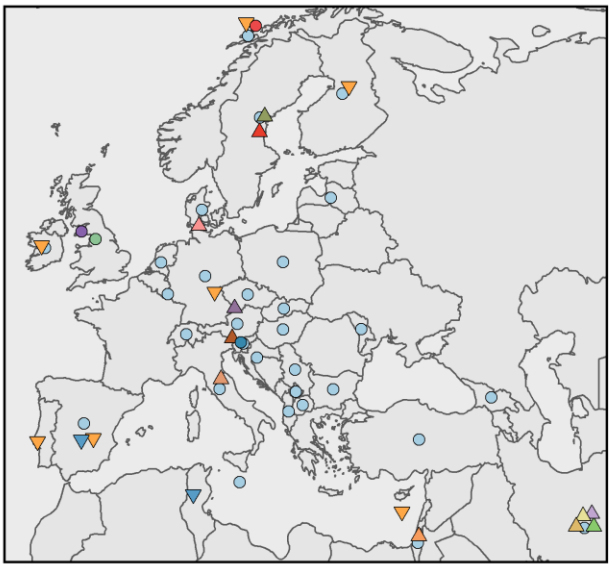
Figure 2: Geographic distribution of wastewater sampling and test approach of included studies

Centroids of countries sampled by included studies are plotted with colours and shapes according to citation and test approach respectively. World Bank regional coverage by study was as follows: East Asia and Pacific (n=1), Europe and Central Asia (n=13), Latin America and the Caribbean (n=2), Middle East and North Africa (n=7), North America (n=1), South Asia (n=1), Sub-Saharan Africa (n=2). Non-Aarestrup et al. 2019 studies are plotted with jitter around the centroid for the map focussing on Europe.





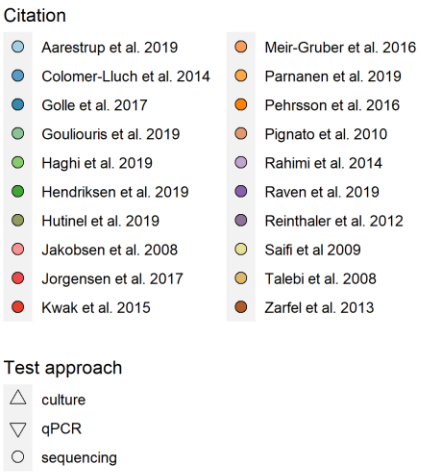


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Figure 3: Interactions between study features in relation to outcome, sampling point and AMR evaluation.

Number of studies with a specific study feature (A=wastewater sampling method, B = longitudinal timeframe, C = reported sewer inputs, D = human dataset used for comparison) plotted against study outcomes, wastewater sampling point and AMR evaluation approach. NAs represent studies without the plotted feature (e.g. snapshot studies for longitudinal timeframe) or did not report feature used. The bar heights represent the number of studies for a given comparison.

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1 **TABLES**

2 **Table 1: Study aims, overview and overall risk of bias**

Citation	Country	Self-defined aim	Targeted organism and resistances (phenotype and genotype).	Relevant comparison	Relevant outcomes	Overall risk of bias
Haghi et al. 2019	Iran	To investigate potential transmission of VRE from wastewater to humans by sampling VRE in wastewater and faecal samples of healthy carriers.	Vancomycin- resistant Enterococcus spp.	Prevalence and resistance patterns of Enterococci isolated from healthy humans and wastewater from the same region during the same time period.	Top three single resistances shared. Wastewater had higher prevalence of resistance to three other antibiotics, multi-drug resistance and VRE.	High
Pärnänen et al. 2019	Portugal, Spain, Ireland, Cyprus, Germany, Finland, and Norway	Trans-European antibiotic resistance surveillance in WwTWs - an initial survey for basing future regular surveillance protocols.	Targeting 229 resistance genes (not organism specific).	The relative abundance of targeted AMR genes in STW influent/effluent collected across multiple European countries compared with AMR surveillance data from the EARS-Net database and national human antibiotic consumption amounts.	Wastewater ARG distribution consistent with country-level gradient from EARS-Net report. Relative abundance of ARG classes reflects country-level antibiotics consumption but more insertion sequences in low-use countries. Effluent antibiotics concentrations and primary care antibiotics consumption not associated to ARG abundance.	Unclear
Hutinel et al. 2019	Sweden	To investigate the relationship between *E. coli* resistance rates in WwTW and hospital wastewater compared to clinical samples.	*E. coli* - no specific resistance targeted.	Resistance rates of *E. coli* isolates from hospital/STW wastewater compared to E.coli isolated from hospital ward and primary care clinical samples during the same time period.	Resistance prevalence of hospital effluent, clinical samples and primary care urine isolates correlated with annual mean resistance rates. ESBLs higher in hospital effluent than WwTW influent. Clinical samples had double resistance rates of influent isolates except for one antibiotic.	Unclear
Zarfel et al. 2013	Austria	To investigate wastewater environmental samples to understand transmission of AMR to humans	Third generation cephalosporin-resistant *E. coli* (*bla*TEM, *bla*SHV, *bla*CTX-M, *bla*VEB and *bla*GES).	AST, biochemical phenotypes, plasmid replicon types and *bla* gene presence of ESBL *E. coli* isolates from STW sludge compared to isolates from hospital patients within STW catchment and collected in the same sampling period	Most prevalent ESBL genes detected were shared across wastewater sludge and UTI isolates. TEM-1 gene detected at similar rates between groups. Both groups shared the same level of resistance pattern diversity. UTI isolates showed reduced phenotypic variation but both groups clustered together with no distinction. Higher resistance rates against clinical antibiotics seen in UTI isolates but not for	Unclear

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Citation	Country	Self-defined aim	Targeted organism and resistances (phenotype and genotype).	Relevant comparison	Relevant outcomes	Overall risk of bias
					non-clinical antibiotics.	
Colomer-Lluch et al. 2014	Tunisia, Spain	To evaluate the abundance of several ARGs in bacteria and bacteriophage DNA derived from wastewater collected from distant sources with differing socio-economic and cultural characteristics, climate and geographic background.	Targeting *bla*TEM, *bla*CTX-M-1, *bla*CTX-M-9, *mec*A, *arm*A, *qnr*A, *qnr*S, *sul*1 (not organism specific).	Abundance of several AMR genes in geographically distant wastewater sources with differing characteristics and historical resistance backgrounds. Country-level comparisons of resistance rates - indirect.	Most common ESBL isolated reflects most reported clinical ESBL. Higher ARG density from high antibiotics use country.	Unclear
Rahimi et al. 2014	Iran	To determine epidemiological relatedness between MRSA strains isolated from wastewater and human infections.	MRSA (mecA).	MRSA isolates collected during the same sampling period from sewage and human clinical infections compared using biochemical fingerprinting, SCCmec typing and ASTs.	Higher rates of multi-drug resistance in wastewater isolates versus clinical. Wastewater isolates also had higher single resistance rates to five other antibiotics. Homogeneity in biochemical typing consistent with contemporaneous outbreaks and dissemination of clones.	Unclear
Jakobsen et al. 2008	Denmark	To investigate the potential spread of gentamicin resistant (GEN-R) *E. coli* isolates or GEN-R determinants from a Danish university hospital to wastewater.	Gentamicin-resistant *E. coli* (*aac*(3)-II, *aac*(3)-IV, ant(2″)-I, *arm*A, *aac*(3)-I, *aac*(3)-III).	Gen-R resistance E. coli isolates from sewage with/out hospital sewer input and from contemporaneous hospital patient UTI samples.	Prevalence of Gen-R *E. coli* similar between hospital effluent and WwTW influent - lowest in residential outlet. No sharing of PFGE types between any patient isolates and wastewater isolates.	Unclear
Saifi et al 2009	Iran	To analyse and compare clonal diversity of gentamicin-resistant *E. faecium* in clinical and WwTW samples to assess release into the environment.	Gentamicin-resistant *E. faecium* (6 ′)-Ie-aph (2 ″)-Ia, aph (2 ″)-Ib , aph (2 ″)-Ic, aph (2 ″)-Id, ant (4 ′)-Ia).	Assessing clonality and resistance prevalence of gentamicin-resistance Enterococcus faecium isolates isolated from clinical and STW samples. Both STWs and clinics in the same city but overlap unclear but associated in time.	Clinical isolate resistance rates higher than wastewater isolates. Individual resistances match national prescribing levels. Low PFGE type overlap between groups.	Unclear
Kwak et al. 2015	Sweden	To provide evidence for AMR wastewater surveillance for monitoring resistance trends and as an early warning system.	*E. coli* (*bla*CTX-M, *bla*TEM, *bla*SHV, *bla*NDM, *bla*KPC).	Resistance rates of *E. coli* isolated from STW influent, effluent and hospital effluent in 2013-2014 compared to national resistance rates of human blood isolates from 2007-2012.	Increasing resistance prevalence of WwTW isolates reflect increase in resistance of human blood isolates. Lower prevalence of resistance in WwTW isolates compared to hospital effluent.	High

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Citation	Country	Self-defined aim	Targeted organism and resistances (phenotype and genotype).	Relevant comparison	Relevant outcomes	Overall risk of bias
Meir-Gruber et al. 2016	Israel	To evaluate the population carriage of pan-resistant bacteria and to establish a convenient and fast method for wastewater sampling.	Carbapenem, methicillin and vancomycin resistance in enterobacteriaceae, *S. auerus and* enterococci spp (*bla*KPC, *bla*NDM-1).	Prevalence of *bla*KPC/*bla*NDM-1 carrying CREs, MRSA and VRE in STW influent and domestic sewer systems collected nationally during 2012-2013 compared to in clinical samples from a major hospital during 2013. Distribution of CREs, MRSA and VRE from sampling sites with and without hospital sewage input.	WwTW samples and clinical isolates shared the same most commonly isolated *bla*KPC-carrying species (K. pneumoniae). They also shared the three other most commonly isolated *bla*KPC species but at different rates. Higher prevalence of MRSA and VRE in hospital wastewater and the receiving WwTWs than in domestic wastewater.	High
Reinthaler et al. 2012	Austria	To assess transmission of antibiotic resistances from human sources into the environment.	*E. coli* - no specific resistance targeted.	Prevalence of antimicrobial resistant *E. coli* and resistance patterns of patient isolates collected in 2000 and 2009 compared to sewage sludge isolates collected during the same time period.	Most commonly isolated resistances shared between patient and sludge isolates but lower overall prevalence in sludge. Increase in individual resistances over time seen in both groups.	Unclear
Talebi et al. 2008	Iran	This study was undertaken to determine the genetic relatedness between the VRE isolates of wastewater and human urinary tract infection isolates.	Vancomycin-resistant *E. faecium* (*van*A and *van*B).	Prevalence of resistance to single/combinations of antibiotics in clinical VRE and wastewater VRE isolates. PFGE and biochemical fingerprinting patterns in clinical VRE compared to wastewater VRE isolates.	MICs higher in human isolates compared to wastewater isolates. Multi-drug resistance detected at the same prevalence between sample groups. Human isolates showed higher PFGE type diversity consistent with no reported contemporaneous outbreaks compared to lower diversity in wastewater isolates.	Unclear
Jorgensen et al. 2017	Norway	To investigate possible transmission routes of ESBL-*E. coli* by comparing isolates from various niches (freshwater lakes, saltwater basins, WwTW and UTIs).	Third generation cephalosporin-resistant *E. coli* (*bla*CTX-M, *bla*TEM, *bla*SHV, *bla*NDM, *bla*KPC).	Genomics of ESBL-*E. coli* isolated from influent compared to those isolated from UTI patients living in the STW catchment area with temporal overlap.	Clinical isolate single and multi-drug resistance rates higher than wastewater isolates. Most prevalent ESBL gene was shared between groups and prevalence for other ESBL genes also similar except M-14 higher in wastewater. Prevalence of acquired resistance genes similar between groups but higher in urine isolates. No difference in plasmid replicon typing between groups.	Unclear
Aarestrup et al. 2019	60 countries sampled	To characterise bacterial resistomes and explain AMR variation in untreated wastewater from sites sampled on a global scale.	Non-limited	Resistomes of sewage influent collected on a global scale compared to source regional antimicrobial use, and socio-economic, health, and environmental factors.	Total AMR gene abundances detected across sites corresponded to existing data describing their country/continent of origin. Country-specific variables made up from World Bank's Health, Nutrition and Population, Development indicator data sets (mostly sanitation and general health)	Unclear

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Citation	Country	Self-defined aim	Targeted organism and resistances (phenotype and genotype).	Relevant comparison	Relevant outcomes	Overall risk of bias
					explained up to 89% of observed resistome variation between samples regardless of diversity of AMR genes. Countries with higher HDI have significantly lower abundance of AMR genes and the number of passengers flying into a country has no effect on abundance. No significant association between temperature at collection with AMR genes abundance. No significant effect was found when investigating total usage of all antimicrobials on the abundance of AMR genes on a class-level. No significant association was found between the abundance of AMR genes on a class-level and the antimicrobial residue levels measured.	
Hendriksen et al. 2019	Kenya	To monitor circulating pathogens and AMR genes via metagenomics of urban wastewater with comparison to other concurrent disease surveillance.	Non-limited	Resistomes of wastewater compared to surveillance data and laboratory-confirmed cases of wastewater source populations.	Increases/decreases in read abundances for bacteria/AMR genes were not reflected in the contemporaneous PBIDS data. Non-significant observed increase in read abundances of pathogens appeared to coincide with reported illness/visits to clinic.	Unclear
Raven et al. 2019	United Kingdom	To evaluate whether the sequencing of *E. coli* isolated from WwTWs could support surveillance of pathogenic *E. coli* lineages and AMR genes in a specific region.	Third generation cephalosporin-resistant *E. coli* with non-limited genotype targeting.	Genomic comparison of STW *E. coli* isolates (with and without hospital sewage input) to *E. coli* blood isolates from hospitalised patients in the same region preceding and during STW sampling period.	Top three most common ST types from clinical blood cultures present in most WwTWs sampled (both receiving and not receiving hospital waste). Most prevalent ESBL gene from clinical samples was detected in all WwTWs. Prevalence of most common ESBL genes approximately reflected between wastewater and clinical samples although higher in wastewater. Majority of AMR gene variants identified were shared between groups. No detection of carbapenem or colistin resistance, consistent with very low resistance to these antibiotics in study area.	Low

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Citation	Country	Self-defined aim	Targeted organism and resistances (phenotype and genotype).	Relevant comparison	Relevant outcomes	Overall risk of bias
Pehrsson et al. 2016	Peru, El Salvador	To map resistance gene dissemination between humans and their environments with focus on low-income populations.	Non-limited	Resistomes and functional metagenomics of human faecal samples compared to wastewater collected from STW serving the human community sampled.	Street-access wastewater and WwTW influent resistomes were equally similar to human-associated resistomes. Street-access wastewater was more similar to influent than human faeces in terms of bacterial composition. All wastewater had higher phylogenetic diversity and more AMR proteins per sample than the human faeces. Drug efflux antibiotic resistance mechanisms were higher overall in human faecal vs wastewater resistomes. Investigating genetic context of cosmopolitan AMR proteins showed network of gene sharing between all sample metagenomes.	Unclear
Golle et al. 2017	Slovenia	To evaluate possible overlap between clinically relevant and environmental strains and determine transfer from wastewater to WWTP.	Carbapenem-resistant *P. aeruginosa* targeting 1411 carbapenemase genes.	Assessing the prevalence/variability of carbapenem-resistant Pseudomonas aeruginosa (CRPA) strains isolated from clinical isolates and STW effluent in the same region using PFGE and WGS.	No pattern or uniformity of PFGE types observed over the year with low overlap (4 isolates) between clinical and effluent samples regardless of region. Low overlap also seen between hospitals and other clinical settings (2). Slightly higher overlap between WwTWs (6). AST-based resistance patterns: clinical isolates have higher diversity in resistance patterns but resistance to individual antibiotics is more prevalent in effluent for 6/8 reported.	Low
Gouliouris et al. 2019	UK	To generate indirect evidence for the extent to which healthcare-associated *E. faecium* is disseminated in the community.	Vancomycin-resistance *E. faecium.* Non-limited genotypes.	Genomic comparison of wastewater *E. faecium* isolates and isolates from clinical infection preceding and during study.	ST typing show high diversity in influent and much lower in clinical samples – consistent with specific ST's association with drug resistance. Comparing relatedness between sample groups showed minimum SNP distance between any bloodstream to wastewater isolates did not differ significantly for plants with hospital input or not.	Low
Pignato et al. 2010	Italy	To investigate whether wastewater isolates can be a useful/easy for	Ampicillin-resistant Salmonella spp. and *E. coli* (*bla*TEM, *bla*SHV,	Prevalence of ampicillin resistance between *E. coli* and *Salmonella* isolates cultured from wastewater compared to	Resistance prevalence in *Salmonella* isolates was of the same order in isolates from wastewater and clinical samples. *Salmonella*	Unclear

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Citation	Country	Self-defined aim	Targeted organism and resistances (phenotype and genotype).	Relevant comparison	Relevant outcomes	Overall risk of bias
		epidemiological monitoring of AMR prevalence in Salmonella clones circulating in the human population.	*bla*PSE-1, *bla*OXA-1 group).	Salmonella isolates cultured from symptomatic hospitalized patients.	resistance profiles were similar between sample sources according to serovar. Specific six-drug pattern observed with a similar frequency in in *Salmonella* serovar *Typhimurium* isolates from wastewater and clinical specimens. Persistent isolation of suspected (not phage-typed confirmed) *Salmonella* DT104 endemic clone accounting for 40% of human salmonella isolates in the study country. Suspected DT104 represented 50% of wastewater isolates but only 22.2% of clinical isolates. Another potential clone with different restricted resistance pattern also appeared to be endemic in the same population.	

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8 **Table 2 - Study methodology**

Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
Haghi et al. 2019	Six sites of "urban sewage waters".	Summer - weather conditions not reported.	Longitudinal samples "collected randomly" from June to October 2017. Unclear timepoints.	Grab samples collected in sterile bottles.	Kept at 4°C until processing - no other details reported.	100 wastewater samples and 100 faecal specimens - total of 141 Enterococci isolated. 77/141 from wastewater samples and 64/141 from faeces.	100 healthy faecal samples with no history of diarrhoea and antibiotic therapy for at least one month.	Disk diffusion with seven antibiotics for ASTs. MIC by agar dilution method.	None conducted
Pärnänen et al. 2019	Influent and effluent from 13 WwTWs from seven countries. Median PE 173839 (IQR: 289906) and flow 33032.5 m3/d (IQR: 100529). Hospital sewer input described for 7/13 WwTWs.	Autumn, spring - weather conditions reported as average annual air temperature based on "timeanddate.com"	Longitudinal sampling with three timepoints over one year (autumn 2015, spring and autumn 2016). Four sites missing timepoints.	24H composite samples (24 hourly sub samples totalling 1-2L) collected on three consecutive days (Tuesday, Wednesday and Thursday).	Filtered (0.22µm) and stored at -80°C before processing.	168 wastewater samples. 142/168 underwent qPCR (92 effluent and 50 influent).	EARS-Net clinical AMR surveillance data from 30 European countries, specifically the 2017 report for 2013-2016 describing "a north-to-south and a west-to-east gradient is evident in Europe".	DNA extraction (PowerWater Kit) and qPCR array with 384 primer sets targeting AMR genes and mobile genetic elements. Culture-dependent detection on selective media (amoxicillin, tetracycline and ciprofloxacin).	PCoA using the Bray-Curtis dissimilarity index.
Hutinel et al. 2019	Influent from WwTW with PE 746,882.	All seasons - weather conditions not reported.	Longitudinal samples collected in 2016 with 1-2 month interval. Up to 12 timepoints.	24H WwTW flow proportional sampling comprised of a minimum of 224 subsamples. 24H hospital effluent composite comprised of	Kept at 4°C and processed within three hours of collection.	14 wastewater samples from which 1252 *E. coli* were isolated.	*E. coli* urine/blood isolates from patients on hospital wards connected to hospital wastewater sampling point or primary care patients from	Microbroth dilution of stocked isolates against 11 antibiotics. ESBL screening by multiple disk diffusion.	Resistance across clinical/sewage modelled with linear regressions.

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Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
				subsamples taken every 9 min (160 subsamples).			municipalities connected to the sampled WwTW (n=6270). Contemporaneous and before wastewater sampling period.		
Zarfel et al. 2013	Activated sludge from five WwTWs "PE range <10,000 to >100,000 and flow rate range 100–1200 L/min".	Winter, spring, summer - weather conditions not reported.	Longitudinal monthly sampling between Feb and July 2009. Six timepoints.	Grab samples collected using sterile wide-mouth bottles.	Kept at 4–8°C for up to 24H before processing.	30 sludge samples from which 50 ESBL *E. coli* isolated.	50 ESBL *E. coli* isolates from hospitalized patients with UTIs collected contemporaneously with wastewater.	AST for 11 antibiotics using microbroth dilution (Vitek). Three additional antibiotics via disk diffusion. CLSI confirmation of ESBL. PCR for five b-lactamase gene families.	None conducted
Colomer-Lluch et al. 2014	Influent from four WwTWs with tourist facility and abattoir sewer inputs (sheep, cattle).	Not reported	Sampling with unclear interval between 2011 and 2014.	Grab samples collected in sterile conditions.	Frozen at -80°C and stored in dry ice before filtration (0.45µm) and processing.	Unclear total wastewater samples: 26 wastewater samples underwent DNA extraction and 28 underwent viral phage fraction analyses.	Country-specific historical resistance rates and specific resistance prevalence.	Samples filtered and phases extracted by QIAamp DNA Blood Mini Kit (retained bacteria) and phenol:chloroform in-house method (phage containing supernatant). qPCR of genes: *bla*TEM, *bla*CTX-M-1, *bla*CTX-M-9, *mec*A, *arm*A, *qnr*A, *qnr*S, sul1. Eight target genes.	None conducted
Rahimi et al. 2014	Urban WwTW west of Tehran.	Not reported	Snapshot samples collected in 2010 - no other details.	Three grab samples collected in sterile 250 ml bottles.	Cold chain transport before filtration (0.45µm) and	Three wastewater samples from which 653 *S. aureus* cultured.	489 clinical samples (wound, urine, sputum, blood, CSF, nose and abscess) from	MIC by E-test for two antibiotics. AST by disk diffusion for 16 antibiotics. PCR detection of *mec*A,	None conducted

Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
					processing.		hospitalized patients collected during the same year as wastewater sampling.	*nuc*A, SCC*mec* (eight types/subtypes) and *ccr* (four types).	
Jakobsen et al. 2008	Influent from WwTW with 230,000 PE receiving industrial hospital (794 bed) input.	All seasons - weather conditions not reported.	Longitudinal monthly sampling between October 2002 - August 2003. 11 timepoints.	24H flow proportional WwTW sampling. Composites (flow-respective mix) of hospital effluent. Composites of residential area outlet comprising 5 samples taken with 10 min interval (5 subsamples).	Not reported	33 wastewater samples from which 55 GEN-R *E. coli* isolates cultured.	38 Gen-R *E. coli* isolates from hospital patients with UTIs during wastewater sampling period with addition month either side.	Microbroth dilution for ASTs to five antibiotics. PCR detection of four genes: *aac*(3)-II, *aac*(3)-IV, ant(2″)-I, and *arm*A. Additional PCR detection of *aac*(3)-I and *aac*(3)-III in subset.	None conducted
Saifi et al 2009	Influent from three WwTWs located in different parts of Tehran.	Not reported	Longitudinal sampling with unclear interval between September 2005 - September 2006. Unclear timepoints.	Not reported	Filtration (0.45µm) - no other details reported.	Unclear total wastewater samples and isolates: total of 106 high-level gentamicin-resistant isolates (clinical = 48, WwTW = 58)	320 enterococcal isolates cultured from urine and wound samples from six outpatient clinics during sampling period.	Disk-diffusion and microbroth dilution for antimicrobial susceptibility testing (10 antibiotics). PCR for five genes (*aac* (6′)-Ie-aph (2″)-Ia, aph (2″)-Ib , aph (2″)-Ic, aph (2″)-Id and ant (4′)-Ia).	None conducted
Kwak et al. 2015	Influent from WwTW with PE >750,000 and flow 250,000 m3/d. Receives	Winter, summer - weather conditions not reported.	Longitudinal sampling with five timepoints over one year: Jan, Feb, July 2013, Jan 2014,	24H WwTW "continuous" sampling via autosampler. 4H hospital composites	Kept at 4°C and analysed within 4H.	17 influent samples from which 1326 *E. coli* isolates cultured. Three effluent samples	Resistance data from EARS-Net European database on human blood isolates limited to	Microbroth dilution (PhenePlate) with 10 antibiotics. Microarray targeting six ESBL and six plasmid-mediated	None conducted

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Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
	hospital input (800 bed) but no significant agricultural sewer input.		Feb.	comprising 50 ml grab samples every 10-15 minutes (16 subsamples).		from which 117 *E. coli* isolates cultured. Six hospital effluent samples from which 451 *E. coli* cultured.	studied country for the years 2007-2012 (preceding wastewater sampling).	AmpC genes.	
Meir-Gruber et al. 2016	Influent from six WwTWs serving large urban areas across Israel (population high = 462670, low = 56943). 4/6 WwTWs received healthcare input (hospitals and nursing homes).	Not reported	Snapshot sampling between Apr 2012 and Nov 2013.	24H WwTW and sewer composites via autosampler (24 or 48 subsamples).	Not reported	Six WwTW influent samples and 10 sewer system samples. Total number of isolates cultured or tested unclear: approximately 560 tested and 112 CRE included in analyses.	All blood cultures, general cultures and rectal swabs received by one major hospital clinical microbiology laboratory in 2013 (semi-contemporaneous) Sample size not reported.	Microbroth dilution (Phoenix) susceptibility testing (antibiotics not provided). RT-PCR for detection of *bla*KPC and *bla*NDM-1.	None conducted
Reinthaler et al. 2012	Activated sludge from five WwTWs with no sewer input from hospitals.	Spring, summer, autumn - weather conditions not reported.	Two sampling periods of six months each divided by ~nine years (five days between Apr - Sep 2000) and (six days between Apr - Sep 2009).	Grab samples using sterile wide-mouth bottles.	Kept refrigerated (4-8°C) for up to 24H before processing.	55 total wastewater samples from which 356 *E. coli* isolates cultured.	Random subset (500) of *E. coli* collection from patient samples (urine, sputum, stool, wound, skin and respiratory tract) taken by general medical practitioners during wastewater sampling periods.	Microbroth dilution (Vitek2) for identification and susceptibility testing to 15 antibiotics.	None conducted
Talebi et al. 2008	Three urban WwTWs located in different areas of Tehran.	Not reported	Snapshot sampling during 2005 - no other details reported	Grab samples using 250 ml sterile wide-mouth bottles.	Kept refrigerated before filtration (0.45 µm) and processing.	Six wastewater samples from which 593 enterococcal isolates cultured.	Enterococcus species isolated from clinical samples (450) (predominately urine) from three major hospitals.	VRE isolated by classical culture-based methods. PCR for detection of *vanA, vanB.* AST disk diffusion and E-test for eight antibiotics.	None conducted

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Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
Jorgensen et al. 2017	Influent from one WwTW.	Summer - weather conditions not reported.	Longitudinal sampling (May 2010, two visits in June, Aug and Sep) - Five timepoints.	24H flow proportional sampling.	Filtered - no other details reported.	Five wastewater samples from which 91 ESBL *E. coli* isolates cultured.	94 patients with community acquired UTI caused by ESBL-*E. coli* living within catchment area of the WwTW collected before wastewater sampling.	Vitek2 microbroth AST and E-test. PCR for *bla*CTX-M and DNA microarray for AMR 11 genes for isolates negative for *bla*CTX-M, Hiseq (150 PE) WGS and analysis using ResFinder and PlasmidFinder.	Univariate and multivariate analyses conducted by binary logistic regression.
Aarestrup et al. 2019	Influent from 53 WwTWs.H15 44/53 received hospital input, 34/53 received industrial input, 17/53 received abattoir input and 14/53 received agricultural input.	Winter, spring summer - weather conditions reported as air temperature at time of sampling and brief description (sunny/cloudy /rainy/foggy/snowy).	Snapshot sampling between 25 Jan and 5 Feb 2016.	24H flow proportional sampling or composites (3 sub samples) over a short time period (minimum 15 min).	Samples frozen at -80°C (48H) before shipping to authors without cold chain. Samples then thawed at 20 for 12H before pelleting via centrifugation and storage at -20 or -80°C before processing.	Samples sequenced for 80 sites (WwTW and others) - results from 79 reported.	World Bank Health, Nutrition, Population, Development indicator data sets collected between the years 2000 and 2016 for 259 countries and territories.	DNA extracted (QIAmp Fast DNA Stool Mini Kit with twice input material and bead beating changes), libraries prepped (NEXTflex PCR-free Library Prep Kit) and sequenced (HiSeq3000 2 x 150bp paired-end). MGmapper used to align against reference sequence databases and AMR genes annotated using ResFinder.	Multilevel Poisson model including observation-level random effects and fixed effects.
Hendriksen et al. 2019	Untreated waste from two confluence points of drainage ditch networks with the high surface flow. Each point drains household	Summer - weather conditions not reported.	Longitudinal sampling every Monday and Wednesday between June 16 and August 26 2014. 42 timepoints.	Grab samples of 500 ml.	Samples whole frozen at -80°C within 2 hours of collection before shipment without coolant to	42 wastewater samples sequenced.	PBIDS local surveillance system comprised of household morbidity and health care usage data contemporaneously collected	DNA extracted (QIAamp Fast DNA Stool mini kit on pellet) and Viral RNA/DNA extracted (Nucleospin RNA XS kit on supernatant). Hiseq and Miseq (PE, 2 x 250bp) used to	Confidence intervals calculated on mean read abundance. Weekly number of reads for specific

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Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
	latrine waste from a separate village: population (population density): 1845 (8.4/m2) and 3727 (8.8/m2).				authors. Samples were thawed for 48H at 4°C prior to processing.		through home visits. Key measures used in study: total cases of diarrhoea, fever, acute febrile illness and number of clinic visits. Stool culture also conducted.	sequence pellet and supernatant extracts respectively. MGmapper used to map against genomic/viral databases and Resfinder for AMR genes.	bacteria, viruses or AMR genes surpassing upper limit defined as significant.
Raven et al. 2019	Influent and effluent from 20 WwTWs where 10 received hospital waste and 10 did not. 15 urban and 5 rural sites. Median PE 47957.5 (IQR 127521) and flow m3/d 27043.2 (IQR 48621.6).	Summer, autumn, winter - weather conditions not reported.	Snapshot sampling between June 2014 and January 2015.	Composite (two subsamples) of consecutive 500 ml samples.	Sodium thiosulphate-based preservation before filtration.	40 wastewater samples from which 394 *E. coli* isolates sequenced. Total of 388 in data analysis after dropping 6 low quality sequences.	Genomes of 437 *E. coli* isolates from hospital patients blood cultures (13/437 overlap with wastewater sampling, 424/437 are prior during 2006-2013).	Microbroth dilution (Vitek2) AST testing/phenotypic carbapenem test. DNA extracted (Qiagen QIAxtractor) and sequenced (Hiseq2000). AMR genes identified with ARIBA and ResFInder. Mobile genetic elements encodings five *bla* genes identified using in silico PCR approach.	Phylogenetic analysis of ST131 based on core genome SNPs.
Pehrsson et al. 2016	Influent and effluent from one WwTW serving population of 700,00 including two shanty towns of low-income families.	All seasons - weather conditions not reported.	Longitudinal sampling between May 2012 and Jan 2013 with 1-2 month interval. Up to nine timepoints.	Samples collected in sterile containers.	Frozen (−20 to −80°C) until shipment to authors where samples were stored at −80°C before filtration (0.22 µm).	24 wastewater samples (12 influent, 12 effluent). Metagenomics on 22 wastewater samples after dropping low-yield extracts. Functional libraries created	Human faecal samples from shanty town serving WwTW sampled - collected semi-contemporaneously with wastewater. 5.7 ± 3.0 individuals per	Metagenomic DNA extracted from pellet via in-house phenol-chloroform bead beating method. Small-insert shotgun expression libraries created (pZE21 in *E. coli* DH10B), colony libraries selectively screened and pooled	Non-parametric Student's t-tests with Bonferroni correction.

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Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
						from 31 human samples from four households. Metagenomic libraries created from 44 human faecal samples.	house sampled. Up to 44 faecal samples analysed.	for DNA extraction. Metagenomic inserts amplified (PCR) and sequenced (HiSeq 2000). Assembly with PARTFuMS and annotation with Resf*arm*s. Metagenomic DNA sheared (300–400 bp) and sequenced (HiSeq/NextSeq 2 × 150 PE). ShortBRED24 used to quantify abundance of AMR genes in the metagenomes based on generated functional metagenomics, CARD, human faecal metagenome assemblies, Lahey b-lactamase database.	
Golle et al. 2017	Effluent from two with distinct community and hospital inputs (1300 or 260 beds). Additional six healthcare facilities including geriatric primary care unit and psychiatric long-term care	All seasons - weather conditions not reported.	Longitudinal sampling between Jan-Dec 2014 with one month interval. 12 timepoints.	Not reported	Refrigerated for up to 6H before filtration (0.45µm).	83 CRPA isolated from effluent samples. Totals including clinical: 208 PFGE types and 112 sequenced.	CRPA cultured during the same sampling period from all respiratory and urine samples from hospitals, other health care facilities and GPs (n=130).	Disk diffusion (10 antibiotics). DNA extraction of subset (QIAamp DNA Mini Kit), MiSeq sequencing and identification of acquired carbapenemase genes using Resfinder.	None conducted

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Citation	WwTW details	Sampling season and weather reporting	Wastewater sampling strategy	Sampling method	Wastewater handling and storage	Wastewater sample sizes	Human dataset	AMR detection method	Statistical tests
	facility). Median flow m3/d 22207.5 (IQR 7928.5).								
Gouliouris et al. 2019	Influent and effluent from 20 WwTWs where 10 received hospital waste and 10 did not. 15 urban and 5 rural sites. Median PE 47957.5 (IQR 127521) and flow m3/d 27043.2 (IQR 48621.6).	Summer, autumn, winter - weather conditions not reported.	Snapshot sampling between June 2014 and January 2015.	Composite (two subsamples) of consecutive 500 ml samples. Hospital grab samples of 1 L.	Sodium thiosulphate-based preservation.	40 STW wastewater samples from which 388 *E. faecium* isolates sequenced. Five hospital wastewater samples from which 40 isolates sequenced. Total of 423 in data analysis after dropping 5 low quality sequences.	187 *E. faecium* associated with bloodstream infection in 187 patients. 140/187 patients in hospitals served by sampled WwTWs preceding wastewater sampling period and 23/187 collected during sampling period.	Selective media for AMR counts and VITEK microbroth dilution for AST. Hiseq for WGS. Phylogenetic WGS analyses with ResFinder (ARIBA) and plasmidSPAdes/*BLA*STN	Maximum-likelihood phylogeny (no recombination/MGE).
Pignato et al. 2010	Influent and effluent from two WwTWs serving separate towns with hospital input for one site. Median PE 21500 (IQR 16000) and flow m3/d 3801.6 (IQR 3110.4)	All seasons - weather conditions not reported.	Longitudinal sampling between Jan-Dec 2004 with 0.5-1 month interval. Up to 24 timepoints.	Composite sampling (10 subsamples).	Refrigerated (4°C) before filtration and processing.	108 total wastewater samples from which 64 *Salmonella* isolates cultured. 273 previously cultured *E. coli* included.	274 Salmonella isolates cultured from faeces of hospitalized patients with gastroenteritis at hospital served sampled WwTW during wastewater sampling period.	Selective media at cut off points to indicate resistance for eight antibiotics. Disk diffusion for subset of isolates (66) against 32 antibiotics to determine resistance patterns. PCR of four *bla* genes and class 1 integron gene cassettes.	None conducted

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Conflicts

The authors report no conflicts of interest

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