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REVIEW

Antimicrobial agent exposure and the emergence and spread of resistant microorganisms: issues associated with study design

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Abstract Antibiotics are essential agents that have greatly reduced human mortality due to infectious diseases. Their use, and sometimes overuse, have increased over the past several decades in humans, veterinary medicine and agriculture. However, the emergence of resistant pathogens is becoming an increasing problem that could result in the re-emergence of infectious diseases. Antibiotic prescription in human medicine plays a key role in this phenomenon. Under selection pressure, resistance can emerge in the commensal flora of treated individuals and disseminate to others. However, even if the effects of antimicrobial use on resistance is intuitively accepted, scientific rationales are required to convince physicians, legislators and public opinion to adopt appropriate behaviours and policies. With this review, we aim to provide an overview of different epidemiological study designs that are used to study the relationship between antibiotic use and the emergence and spread of resistance, as well as highlight their main strengths and weaknesses.

Introduction: setting the scene

Antibiotics are essential agents in human medicine. Along with hygiene and vaccination, their discovery in the 1940s changed the patterns of major human diseases, ending the era of 'infectious diseases' and beginning the era of chronic and degenerative diseases [1–3]. This epidemiological transition [1] led to a decrease in infantile mortality and increase in life expectancies. However, we are now facing a new transition involving the re-emergence of infectious diseases and specific concerns regarding resistant pathogens [3]. The use, and sometimes overuse, of antibiotics in humans, veterinary medicine and agriculture have provided selective pressure favouring the emergence and spread of resistant microorganisms [4].

Antibiotic use in human medicine might have serious implications at both individual and population levels (Fig. 1). It might initially increase a patient's risk of colonisation or infection with resistant organisms. Under selection pressure, commensal or pathogenic microorganisms might acquire new resistance mechanisms and pre-existing resistant microorganisms might increase. In addition, the colonisation resistance of commensal flora will be impaired, leading to increased susceptibility to colonisation with new and resistant microorganisms. Antibiotic use may also increase the risk of colonisation or infection with resistant organisms in people who have not received antibiotics. Indeed, the increased reservoir of resistant organisms among antibiotic users will disseminate into the community, increasing the probability that others will acquire resistant microorganisms [5].

However, if the impact of antimicrobial use on resistance is intuitively accepted, the general public, physicians and legislators require scientific rationales in order to understand the consequences of overprescription, change their practices and adopt suitable policies. This review provides an overview of the different study designs used to establish the relationships between antibiotic use and the emergence and spread of resistance; further, it highlights their main strengths and weaknesses.

Establishing causal inference in epidemiology

Establishing causal inferences [6] is part of the general process of scientific reasoning. In epidemiology, different models of causation are used to investigate risk factors

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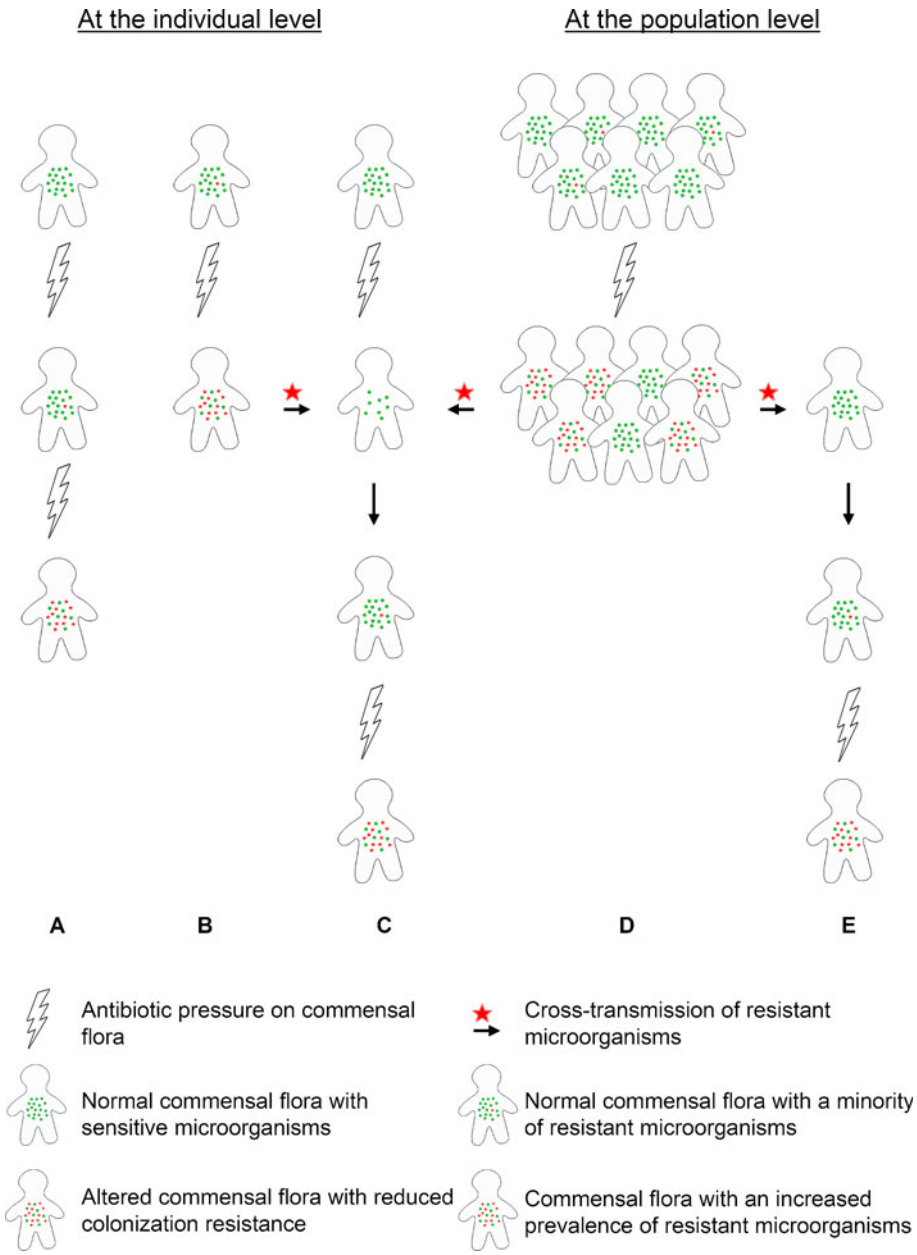

Fig. 1 Role of antibiotic pressure on the emergence and spread of resistant microorganisms in commensal microflora at the individual and population levels. *A* Acquisition of new resistant mechanisms among commensal flora under antibiotic pressure. *B* Rising density of colonisation by resistant microorganisms already present at low levels in commensal flora under antibiotic pressure. *C* Impairment of the normal flora and colonisation resistance associated with antibiotic pressure, leading to increased susceptibility to colonisation by new resistant microorganisms. *D* At the population level, the combined consequences of individual antibiotic treatments might lead to an increased reservoir of individuals who are colonised by resistant microorganisms that are likely to disseminate into the community. *E* Possible cross-transmission of resistant micro-organisms emerged among the commensal flora of antibiotic users to unexposed people

associated with medical events [6–9]. Different sets of criteria have been proposed to assess causality, although none are infallible [10–13]. However, most epidemiologists define three main criteria of contributory causes: (i) the existence of an association between a cause and an effect at an individual level; (ii) the respect of temporal sequence (i.e. the cause precedes the effect); and (iii) the reversibility of the effect (i.e. altering the cause alters the probability of occurrence of the effect) [8]. Other criteria such as the strength and consistency of the association or dose–response relationship may also contribute to defining contributory causes (Table 1) [8, 11, 14].

The findings of epidemiological studies are of particular interest because they can lead to better understanding of diseases and create new strategies for prevention. In our field, many studies have been conducted in order to highlight the risk factors associated with the emergence of resistant microorganisms; their findings have prompted calls to reduce unnecessary antibiotic use, enhance surveillance systems, facilitate antimicrobial development and alert public opinion [15–22]. However, the 18th-century empiricist, David Hume [10], stated that certainty is impossible to obtain in empirical sciences; even the most carefully designed epidemiological study may be controversial or irreproducible [14]. Indeed, all studies potentially face problems demonstrating their internal or external validity (Table 2). Internal validity can be defined as the ability to avoid systematic errors (i.e. biases) and truly measure what was intended. All observational and some experimental studies present built-in biases such as selection, information

Table 1 Criteria for assessing the evidence of causality

Three major criteria for establishing the existence of a contributory cause:

- The existence of an association between a cause and an effect at the individual level.
- The respect of a temporal sequence: *The cause precedes the effect.*
- The reversibility of the effect: *Altering the cause alters the probability of the occurrence of the effect.*

Other commonly used ancillary criteria:

- The consistency of the association: *Is the same association found in many studies?*
- The strength of the association: *Quantitatively measured through statistical tests (e.g. odds ratios, relative risks, p-values).*
- A dose–response relationship.

and confounding biases that undermine the internal validity and quality of their results [23, 24]. In contrast, external validity is the extent to which the results of a study can provide a correct basis for a further generalisation (i.e. generalisability) [23, 24]. It is critical that clinical researchers, microbiologists and epidemiologists, studying the association between antibiotic exposure and the risk of the emergence of resistance, design robust studies that minimise internal biases and potential threats to the extrapolation of their findings.

Observational studies

Observational (i.e. non-experimental) studies describe the size and direction of a relationship among variables, including individual characteristics such as demographics or medical data, without implying any intervention from the investigator [25, 26]. They are frequently used because they are generally easier to implement than experimental studies [26–29]. However, unlike interventional studies, they are unable to demonstrate the reversibility of an effect and assess all criteria of a contributory cause (Table 1).

Cohort studies

Cohort studies follow up and compare different groups of subjects undergoing various exposures (e.g. antibiotic treatment vs. no treatment). However, unlike experimental studies, investigators select subjects to be observed and classify them according to the exposure status, rather than by assigning them to exposure groups [26]. In most cases, cohort studies prospectively follow volunteers until endpoints or the end of the study is reached. In our field, follow-up periods are not very long (a few weeks to a few months) because of the short duration of antibiotic courses, rapid emergence of resistance under antibiotic and limited length of stay of hospitalised patients [30–35]. Two other kinds of cohort also exist: retrospective cohorts, in which investigators identify subjects reaching the endpoint and where medical records are used to collect data about exposure [36–38], and ambidirectional cohorts, in which data collection goes in both directions [39, 40].

Due to their special design, cohort studies have appealing advantages. Since they collect information from exposition to outcome, they offer the possibility of analysing a clear temporal sequence between the putative cause and outcome, which is ideal for demonstrating causality (Table 1) and allowing the calculation of the incidence rate. In our field, prospective designs also allow the planning of serial surveillance sampling screening for colonisation with resistant microorganisms (as opposed to clinical sampling screening for infection) before, during and after antibiotic therapy. This is very useful for determining the acquisition of resistant microorganism in commensal flora [5] and has been

Table 2 Assessing the quality of epidemiological studies

Internal validity: *Is the study able to avoid systematic errors (i.e. biases)?*

- Selection bias: The groups compared should be similar in all important aspects except for the exposure (i.e. cohort study), outcome (i.e. case–control study), intervention and treatment (i.e. quasi-experimental study or randomised control trial).
- Information bias: A systematic error introduced during the process of obtaining information regarding exposure or the outcome of interest. This includes recall and reporting bias.
- Confounding factor: A bias due to a variable distributed differently in the study and control groups that will affect the outcome or exposure being assessed.

Observational studies are particularly susceptible to internal validity issues.

External validity or generalisability: *Are the results of the study able to provide a correct basis for further generalisation to other populations or settings?*

Interventional as well as observational studies are susceptible to external validity issues.

used in several studies [30, 32, 33, 35, 41]. D'Agata et al. followed a cohort of patients undergoing haemodialysis through serial rectal swabbing from hospital admission until discharge and found that the acquisition of vancomycin-resistant enterococci is associated with vancomycin exposure [30]. Cohort studies can also help evaluate the risk of clinical infection once colonisation with resistant microorganisms has occurred [5]. Razazi et al. carried out a prospective study focusing on extended-spectrum beta-lactamase-producing Enterobacteriaceae acquisition and further infections associated with such bacteria among intensive care unit patients [42]. More generally, cohort studies are useful because they allow the calculation of true relative risks in contrast to case–control and cross-sectional studies, which only enable relative risk approximation. Finally, cohorts might also be useful for investigating multiple outcomes associated with a single exposure [8, 26, 43], such as the emergence of different resistant microorganisms under a unique antibiotic pressure [36].

However, like all observational studies, cohorts have limitations that threaten their internal validity (Table 2). First, selection bias can be an issue. Indeed, cohorts should theoretically compare groups of subjects similar in all respects except for the exposure and, therefore, the choice of the control group is critical. Internal comparison (e.g. controls chosen among patients from the same ward not receiving antibiotics) is always preferable for minimising the baseline differences between groups. However, the exposition criteria themselves might lead to substantial differences between groups. For example, patients receiving broad-spectrum antibiotics might not be comparable to patients receiving narrower-spectrum antibiotics or no antibiotics. They might have a more severe condition perhaps associated with an increased number of past hospitalisations that is potentially responsible for an increased load of multi-resistant organisms. Second, cohorts may also suffer from information bias, i.e. errors associated with the collection of the information used to measure the outcome (Table 2) [8, 23, 26, 44]. To limit such biases, outcomes should be defined clearly and information questionnaires standardised [23].

Another problem is confounding factors [8, 23, 26, 44]. Confounding factors are variables distributed differently between exposed and unexposed subjects that will affect the outcome but are not intermediate links in the chain of causation (Table 2) [8, 23, 26]. They can be random or due to selection bias and obscure the causal relationship examined by the study [8, 23, 26]. However, there are strategies to limit confounding factors. Individuals with known confounding factors can be excluded at inclusion, although this can potentially hinder the recruitment process and further generalisability of the study (Table 2). It is also possible to apply a correction for confounding factors during multivariate analyses [8, 23, 26].

In contrast, loss to follow-up is a specific issue in cohort studies. It has two main consequences: (i) it can diminish the power of the study and (ii) it can introduce a bias, distorting results due to different bail-out rates in the exposed and unexposed groups [43]. The best way to avoid loss to follow-up is to pay attention during enrolment and include only participants who will probably complete the study. Conversely, such a strict attitude also has downsides; it can generate selection bias, hinder recruitment and impair the further generalisability of the results. However, in our field, loss to follow-up is not a substantial problem, as follow-up periods are usually short. Another major limitation associated with follow-ups in cohorts are the costs and difficulties of implementation, which often discourage researchers and explains why these observational designs are used less frequently than others (Table 3).

Case–control studies

Case–control studies are popular because of their appealing advantages. First, they require relatively less time, because the event of interest has already occurred when the study starts [26, 44]. Second, they are useful when studying rare conditions such as resistant microorganism infection [45]. Third, they allow the simultaneous investigation of multiple exposures associated with the outcome (e.g. multiple antibiotic exposures) [26, 44]. Their main features are to identify individuals who present with a medical event of interest (e.g. colonisation or infection with a resistant microorganism) from a well-defined source population and select controls representative of the source population who do not present with the event [8, 26, 44]. The control group will allow the evaluation of the exposure distribution within the source population and, subsequently, compare it to that observed in cases [26]. This will allow the calculation of odds ratios, which are a good estimate of relative risks when the incidence rate of the outcome is low in the population [46]. There are several papers in the literature that address the methodological issues of case–control studies on antibiotic risk factors associated with antibiotic-resistant organisms [45, 47–51].

The first issue is the choice of question to be addressed (i.e. the impact of antibiotic exposure on the acquisition of resistant microorganisms or on the risk of infection) and of the definition of the outcome [48]; most investigators use the results of clinical cultures to define their cases, whether they address the former [52–55] or the latter [56–59] question. If this makes sense when studying the post-antibiotic risk of infection, when studying acquisition, it may lead to the identification of distorted risk factors such as risk factors associated with the current infection, regardless of the presence or absence of resistant microorganisms [48]. Moreover, when using clinical samples to study community patients,

Table 3 Main characteristics, strengths, challenges and limitations of different study designs investigating the causal relationships between antibiotic exposure and the emergence and spread of resistant microorganisms

	Feature	Strengths	Challenges	Limits
Observational studies:				
Cohort study	Prospective study following two groups of subjects undergoing different exposures (e.g. antibiotic vs. no antibiotic use) and comparing the requency of the outcome (i.e. the emergence of resistant microorganisms in colonisation or infection samples) in both groups	▪ Respect of temporal sequence ▪ Causal association established using relative risk ▪ Possibility of exploring multiple outcomes for one exposure ▪ Useful for investigating rare exposures ▪ Serial surveillance can help distinguish between the acquisition of and infection with a resistant microorganism	▪ Establishing a strategy to limit loss to follow-up, which impairs the power of the study and possibly introduces bail-out bias ▪ Minimising biases threatening internal validity – Selection bias: are non-exposed con trols comparable to exposed volunteers? – Information bias: use of appropriate techniques for data collection and outcome definition – Strategy to deal with confounding factors	▪ Unable to test the reversibility of the effect ▪ Time consuming ▪ Difficult to implement ▪ Expensive
Case–control study	Retrospective study comparing past exposures (e.g. antibiotic use) in a group of individuals colonised or infected with resistant microorganisms to past exposures in a non-colonised or non-infected group	▪ Requires relatively little time ▪ Possibility of exploring multiple exposures ▪ Useful for investigating rare events ▪ Inexpensive	▪ Choosing the appropriate control group: are controls carrying the susceptible microorganism of interest or free of the microorganism of interest? ▪ Minimising biases threatening internal validity – Selection bias: matching of cases and controls – Information bias: use of appropriate techniques for data collection – Strategy to deal with confounding factors	▪ Difficult to establish temporal sequence ▪ Unable to test the reversibility of the effect ▪ Causal association established through odds ratios, a good estimator of relative risk, when the incidence rate of the outcome is low in the population
Cross-sectional design	Point prevalence study simultaneously assessing the status of the volunteers with respect to colonisation or infection with resistant microorganisms and their past exposures in order to compare them	▪ Requires relatively little time to conduct ▪ Possibility of exploring multiple exposures ▪ Inexpensive	▪ Minimising biases threatening internal validity – Information bias: the use of appropriate techniques for data collection – Strategy to deal with confounding factors	▪ Difficult to establish temporal sequence ▪ Unable to test the reversibility of the effect ▪ Difficult to minimise selection bias ▪ Causal association established through odds ratios, a good estimator of relative risk, when the incidence rate of the outcome is low in the population
Interventional studies:				
Randomised controlled trials	Prospective studies randomly assigning healthcare intervention (e.g. one antibiotic treatment vs. another) in volunteers and evaluating the effect of the intervention on an outcome of interest (e.g. the emergence of resistant microorganisms)	▪ Gold standard of causal research in medical sciences ▪ Randomisation allows the comparison of probabilistically similar groups ▪ Demonstration of the three criteria of a contributory cause: – Establishing association between cause and effect	▪ Respect of ethical considerations ▪ Implementation of the randomisation process: choosing a strategy to keep the allocation sequence concealed ▪ Using a blinding strategy to avoid detection bias: a microbiologist analysing samples should not be aware	▪ Possible lack of external validity ▪ Difficult to implement ▪ Ethical limitations ▪ Expensive

Table 3 (continued)

	Feature	Strengths	Challenges	Limits
		– Respect of temporal sequence – Assessing the reversibility of the effect ▪ Serial surveillance can help distinguish between the acquisition of and infection with resistant microorganisms	of the treatments received by the volunteers	
Quasi-experimental design	Prospective studies assigning healthcare intervention without randomisation in groups (e.g. antibiotic treatments in hospital wards) and evaluating the effect of the intervention on the outcome (e.g. the emergence of resistant organisms).	▪ Respect of temporal sequence ▪ Assesses the reversibility of the effect ▪ Easier to implement than randomised controlled trials	▪ Choosing an appropriate design to control for confounding factors: designs with control groups and pre-test meas urements to assess the initial comparability ▪ Choosing an appropriate design to deal with maturation effects and demonstrate the reproducibility of the results over time	▪ Possible lack of internal validity due to the comparison of non-randomised groups ▪ Possible lack of external validity ▪ Difficult to implement higher-quality quasi-experimental designs
Population-level and multi-level studies	Observational or interventional (cluster-randomised trials) designs aiming to study causal relationships between antibiotic exposure at the population level and the emergence or spread of resistance	▪ Major way of describing the indirect effects of antibiotics ▪ Evaluation of the link between changes in antibiotic prescription and the level of antibiotic resistance ▪ Cluster-randomised trials can be imple mented to test health intervention when individual-randomised trials are unethi cal or impractical	▪ In observational designs, the collection of full information regarding antibiotics consumption at the population level is an issue. The best option is to combine aggregated data and individual data ▪ In cluster-randomised trials, the anal ysis will be considerably more complex than that in individual-randomised trials because the data are not independent	▪ Possible lack of internal validity in observational studies ▪ Cluster-randomised trials require large sample sizes to be effective, making them difficult to implement
Systematic reviews and meta-analysis	Systematic review of studies focusing on a topic of interest (e.g. the impact of the antibiotic exposure and the emergence of resistance) associated with a combined analysis of the data reviewed	▪ Calculation of more precise estimates of the risk factor effects ▪ Generalises the results of single studies ▪ Reconciles studies with discordant results ▪ Assesses weak risk factors that have large impacts on public health ▪ Investigates risks associated with rare medical events	▪ Developing the most accurate research protocol to avoid combining non-comparable results (of particular con cern with observational study results): – Importance of the evaluation of inter-study heterogeneity – Re-analysis of individual data in a new common model instead of combin ing published data	▪ Time consuming ▪ Publication bias
Mathematical models	Use of a theoretical framework to conceptualise and investigate the association between antibiotic exposure and resistance patterns and under various circumstances	▪ Generates and tests hypotheses about the relationships between antibiotic exposure and resistance under various conditions ▪ Prioritises factors to control in order to prevent resistance	▪ Choosing an appropriate model to address the questions of interest (e.g. small populations and random variability)	▪ Modelling implies simplification; models cannot truly address the level of complexity of reality

there is a risk of using samples collected only because the first-line antibiotics failed [60], which might result in an overestimation of the association between resistance and prior exposure [60]. Hence, surveillance cultures assessing the colonisation status of patients are preferable for addressing the acquisition question [48].

Another challenge lies in the selection of the control group. Indeed, if the level of exposure in the controls is not representative of the actual baseline in the source population, selection bias will occur, threatening internal validity (Table 2) [23, 46]. To limit this phenomenon, investigators generally match cases and controls on the basis of criteria such as age, gender, hospital ward and, in our field, the time at risk for acquisition (i.e. the length of hospital stay) [49]. However, the matching process has drawbacks; it can hinder recruitment and, by definition, it is impossible to examine the effects of matched variables [23]. Investigators studying the acquisition question must face another challenge associated with the choice of controls [47–49, 51]. If the study aims to determine the risk factors for acquiring a specific resistant microorganism, controls should be chosen from among patients with negative cultures for the causative microorganisms [49]. However, in their review of 37 case–control studies, Harris et al. state that investigators most frequently select controls from among patients with positive cultures for susceptible microorganisms [47]. Consequently, they might improperly identify antibiotics as risk factors for acquiring resistant microorganisms. Indeed, antibiotics have intrinsic activities against susceptible organisms, and controls defined as carriers of susceptible organisms are less likely to have been exposed to antibiotics than the source population or cases [47]. In contrast, if the study aims to establish risk factors for developing resistant strains in individuals already carrying susceptible strains, controls should be chosen from among subjects with positive cultures for the susceptible microorganism [49].

More generally, case–control studies might present other issues related to internal validity (Table 2) [8, 23, 26]. First, information biases can occur; they involve recall and reporting biases, which arise when subjects from one group are more likely to recall or report events than others, as well as classification or measurement biases, which result from dissymmetric information collection according to a subject's status as a case or control [8, 23, 26]. All of these biases may impair the quality of risk factor measurement and distort associations between exposure and outcome. To reduce these biases, information about expositions should be gathered by interviewers blinded of the subjects' case or control status using standardised questionnaires [8, 23, 26]. Second, investigators must deal with the recurrent issue of confounding factors [8, 23, 26]. As described above, different strategies exist to minimise confounding factors, including excluding individuals with known confounding factors, matching processes and mathematical correction [8, 23, 26] (Table 3).

Cross-sectional studies

Cross-sectional or prevalence studies include all people or a representative sample from a population, regardless of exposure or outcome [26]. They are performed to examine the presence or absence of a medical event such as carriage or infection with a resistant microorganism and exposure to an antibiotic at a particular time [26, 27]. Similar to case–control studies, they are frequently used because they require relatively little time and are easy to implement [8, 26, 60–63].

However, cross-sectional studies have critical limitations. Since both exposure and outcome are ascertained simultaneously, the temporal sequence cannot be clearly established (Table 1) [27]. For example, if colonisation with resistant microorganisms is more frequent among subjects exposed to antibiotics, does it mean that prior antibiotic exposure leads to extra colonisation or that subjects with higher colonisation consume more antibiotics because they are more prone to infections? Another problem, called length-biased sampling, is the over-representation of cases with chronic conditions [27]. For example, subjects with a transitory excess of resistant microorganisms following antibiotic exposure are less likely to be identified as cases than subjects carrying long-term resistant microorganisms due to an underlying medical condition. Finally, cross-sectional studies are susceptible to other internal validity threats such as information biases due to their retrospective nature and confounding factors (Table 2) [8, 23, 26]. Therefore, they might not be the optimal study design for achieving aetiologic objectives (Table 3).

Interventional studies

A common attribute of interventional studies is the control of one or more variables, manipulated by the investigator, to observe their effect on dependant variables [25, 64]. Interventional studies offer the possibility to test the reversibility of an effect (Table 1). Moreover, the assignment of exposure by the investigator is a way to limit confounding factors and enhance internal validity (Table 2) [26].

Randomised controlled trials

Randomised experiments are considered to be the gold standard of causal research in medical sciences and are at the top of the hierarchy of study types in the evidence-based literature [25, 65–67]. They are prospective studies in which

volunteers are randomly assigned healthcare interventions. If designed well, these studies are able to demonstrate all three criteria of a contributory cause (Table 1). This is why they gained increasing recognition during the 20th century and became the best approach for assessing healthcare interventions. However, few randomised trials have been designed to investigate the association between antibiotic exposure and the emergence or spread of resistant microorganisms [68–74]. In contrast, several trials have been designed to evaluate the efficacy of antibiotics, while some analyse resistance as a secondary outcome [75, 76].

Randomisation is the key principle in randomised controlled trials. The random assignment of treatments to the participants, which is controlled by the investigators, allows the creation of probabilistically similar groups. Hence, any differences observed between groups are probably due to differences in the treatments [25]. In other words, proper randomisation reduces the selection and confounding biases (Table 2) that hinder all other epidemiological studies [23, 77]. However, it is important to stress that randomisation is associated with critical implementation issues [77, 78]. There are many methods for generating unpredictable randomised allocation sequences—some as simple as tossing a coin or using a random number table. However, their benefits might be undermined if the allocation sequence is not kept concealed [24, 77, 78]. Knowledge of the next assignment could cause selection biases to seep back into trials; investigators could exclude patients perceived to be part of an inappropriate group [24, 78]. Schulz et al. demonstrates that trials using inadequate allocation concealment strategies yield larger estimates of treatment effects and produce more heterogeneous results [79]. The generation and concealment of unpredictable randomised allocation sequences are the keys to ensuring the unbiased nature of randomised trials.

However, randomised trials are sometimes susceptible to other systematic errors. First, detection biases can occur when the investigators assessing the outcome know the treatment that has been allocated to the participants. Blinding strategies, in which the nature of the treatment is masked to the participants and/or investigators, can be used to prevent this [80, 81]. In our field, microbiologists analysing samples for resistance should be blinded to the treatment of the participants [71]. Another possible difficulty, similar to cohort studies, is the loss to follow-up [43, 81]. However, in our field, this problem is generally minor, because follow-ups do not last very long (1–2 months in the above-mentioned studies) [68–74]. Finally, although randomised trials may be the best option for ensuring high internal validity, they can suffer from a lack of external validity (Table 2). Indeed, inclusion and exclusion criteria are sometimes unduly rigorous, leading to the exclusion of particular at-risk groups [27]. In addition, patients willing to participate tend to differ from those who choose not to participate [27]. Both factors might limit the generalisability of the findings of such studies [27] (Table 3).

Quasi-experimental studies

Quasi-experimental studies aim to demonstrate causality between an intervention and outcome without using randomisation [25, 82, 83]. Even if their credibility for assessing causality is lower than that of randomised trials, they are frequently used when it is not logistically feasible or ethical to conduct randomised trials [25, 82, 83]. In our field, they are invaluable for evaluating the impact of antibiotic prescription control policies with respect to resistant microorganisms and assessing the performance of interventions implemented to limit outbreaks of resistant microorganisms. There are various papers in the literature that address their methodological issues in the fields of infection control and antibiotic resistance [82–86]. As described by Harris et al. in 2004 and 2005 [82, 83], there are many existing designs for quasi-experimental studies that can be clustered into three groups: (i) designs that do not use control groups, the most frequent being the one-group pre-test–post-test (before-and-after) design, in which investigators make measurements in a unit/hospital before the intervention and another afterwards [87–94]; (ii) designs that use control groups but no pre-test, in which investigators apply an intervention in a unit/hospital and compare the outcome to an intervention-free unit/hospital [95]; and (iii) designs that use control groups and pre-test, most often using one group with an intervention and another intervention-free group, with measurements before and after the intervention [96]. Harris et al. propose a hierarchy of these designs with respect to their internal validity quality: group (iii) is better than group (ii), which is better than group (i) [82]. However, they report that higher-quality designs are not used most frequently probably because they are more difficult to implement [83]. This is corroborated by de Bruin and Riley, who analysed 12 quasi-experimental studies on the effect of vancomycin-prescribing interventions on vancomycin-resistant enterococci and found all of them to be simple pre-test–post-test designs [84].

Even though quasi-experimental studies meet the three major criteria of contributory causes (Table 1), they present limitations arising from non-randomisation, leading to difficulties in controlling for confounding factors [82, 83]. The best way to minimise threats to their internal validity is to use higher-quality designs that include control groups, which may help identify confounding factors, and pre-test measurements, which allow the assessment of the initial comparability of groups and highlight potential selection bias [82, 83, 85].

Another limitation of quasi-experimental studies is ‘maturation effects’ [82]. Indeed, measurements before and after

an intervention may be separated by several months, and observed variations might simply be the result of seasonal cycles. To limit this problem, investigators can choose a higher-quality study design, in which the intervention is first administered to the intervention group and then to the control group. This helps demonstrate the reproducibility of the results in the two groups at two different times (Table 3).

Population-level and multi-level studies

Previous designs gathered individual-level data and studied the direct effects of antibiotics on resistance. In contrast, population-level studies are a common way to describe the indirect effects of antibiotics on resistance (Fig. 1) [5]. These designs are very useful for establishing links between changes in the levels of antibiotic prescription (e.g. reduced antibiotic use following local or national recommendations) and antibiotic resistance [97]. Among population-level studies, investigators can choose between observational or cluster-randomised interventional studies.

In population-level observational studies, aggregated data regarding antibiotics are obtained either out of reimbursement data or through sales or distribution data [97–100]. Unfortunately, both strategies present limitations: using reimbursement data is associated with a risk of under-detection bias due to unaccounted over-the-counter sales, parallel trade or non-reimbursed antibiotics, while using sales/distribution data is associated with risks of under- or over-detection bias due to parallel import or export [100]. Therefore, population-level studies sometimes lack sufficient data regarding antibiotic exposure to avoid confounding factors [101], and studies that estimate both individual- and population-level antimicrobial use (i.e. multi-level studies) are preferable [5, 99, 102, 103]. For instance, Harbarth et al. demonstrate this in a study reporting a significant association between antibiotic exposure and resistance at the individual level but not at the group level [102]. Similarly, Donnan et al. studied the association between trimethoprim resistance in urinary bacteria and antibiotic exposure and found no association at the practice level, whereas individual exposure to antibiotics was significantly associated with trimethoprim resistance in the multi-level model [99].

The main feature of cluster-randomised trials is the same as that of a randomised trial, except that investigators control the intervention by assigning it randomly to groups of patients rather than individuals [104–106]. Cluster-randomised trials are a key tool for evaluating interventions at the group level, especially in health services [104–106]. They can be implemented when it is impractical or unethical to randomise individuals. However, they also present noteworthy drawbacks. First, many more subjects are required to obtain the same level of precision [44]. Second, it is considerably more complex to analyse the data produced because they are no longer independent and basic statistical procedures generally assume such a characteristic [44]. Therefore, cluster-randomised trials remain rare in our field. We identified less than ten studies using the search terms ‘cluster randomised trial’, ‘anti-bacterial agents’ and ‘resistance’ in the MEDLINE database [107–110]. The work of Skalet et al. focusing on trachoma control in Ethiopia and re-analysed to discuss *Streptococcus pneumoniae* resistance [109] is an interesting example; 24 communities were randomised to receive either azithromycin mass treatment during the first year of life or no antibiotics. This work demonstrates that frequent azithromycin use selected for macrolide resistance among nasopharyngeal *S. pneumoniae* at the community level. The work of de Smet et al. also provides an example [107, 108]. Patients with mechanical ventilation in 13 intensive care units in the Netherlands were randomised and received standard care, oropharyngeal decontamination with topical antibiotics or digestive decontamination with topical antibiotics plus intravenous cefotaxime. The results show that the acquisition of respiratory tract colonisation with highly resistant pathogens is lowest with the digestive decontamination procedure [108] (Table 3).

Systematic reviews and meta-analysis

Until the mid-1990s, the system for ranking the levels of evidence in clinical research attributed the highest grade to ‘at least one properly randomised controlled trial’ [111, 112]. However, this paradigm has been challenged by different studies showing that well-conducted observational studies can produce results similar to those of randomised trials [67, 113, 114]. Meanwhile, the results of randomised trials can exhibit significant discrepancies possibly because they are less likely to include a broad representation of the population [67, 115]. These findings led to the conclusion that a single randomised trial cannot provide gold-standard results on a topic of interest and that evidence from different studies should be analysed together [65, 67].

Thus, researchers perform systematic reviews associated with a meta-analysis (a combined analysis of the data reviewed). This approach aims to identify, synthesise and, if possible, quantitatively combine the results of relevant studies. Meta-analyses increase statistical power and allow the calculation of more precise estimations of the effects of risk factors. It is of great interest to generalise the results of single studies, reconcile inconsistent results, assess weak risk factors with large impacts on public health (as in our field) and investigate the risks associated with rare diseases [116]. Systematic reviews and meta-analyses have been increasingly used over the past decade, primarily for randomised controlled trials and observational studies to a lesser

extent [67, 117–120]. However, few focus on the relationship between antibiotic exposure and resistance. We found less than ten studies in the MEDLINE database using the search terms 'meta-analysis', 'anti-bacterial agents' and 'resistance' [60, 84, 121–124].

However, the strength of meta-analyses may also be their weakness, and two major difficulties arise from combining and generalising the results of different studies. The first problem is publication bias: studies with negative results are less likely to be published than others. This limitation could lead the meta-analysis to overestimate the relationship between an exposure and outcome [116, 117, 125]. Second, not all included studies might have the same degree of quality and they might possess various biases. Hence, combining their results could lead to the combination of non-comparable results [117]. This limitation is particularly important when combining the data of observational studies, which are more likely to be biased. The best way to minimise this issue is to thoroughly develop and conduct the research protocol. First, the most accurate research question should be defined using common sense, clinical insight and biological plausibility. This will aid the selection of precise inclusion and exclusion criteria to select relevant studies [125, 126]. After the selection process using predetermined search strategies in electronic databases such as MEDLINE, the validity and comparability of included studies must be evaluated through statistical tests assessing the magnitude of inter-study heterogeneity [116, 127–129]. This point is crucial because it will determine whether a pooled estimate can be calculated or not. If the heterogeneity is high, no overall estimate should be reported. However, this problem can be partly avoided if the reviewers have access to the individual data from each study instead of the overall data. In this case, all data will be re-analysed using the new statistical model, thus, presenting the results in a unified way. Unfortunately, this type of meta-analysis is very rare because it is more expensive and very time consuming (Table 3).

Mathematical modelling

Mathematical modelling is another tool for analysing the associations between antibiotic exposure and the emergence and spread of resistance at the individual or population level [5]. Modelling implies the use of a theoretical framework and aims to explore scenarios under varying conditions [130, 131]. Two kinds of models are found in the literature. The oldest ones are deterministic models using ordinary differential equations [130, 131]. They are applied when the dynamics of the study population can be described according to the average behaviour of individuals [130, 131]. Individuals are aggregated into homogeneous compartments (e.g. colonised/non-colonised or infected/non-infected), the parameters of the model are fixed and chance events are not taken into account. Unfortunately, these models are not well suited for small populations such as hospital wards, in which fluctuations of colonisation or infection with resistant organisms might happen by chance [130, 131]. To take into account the random variability of the outcome, modellers use stochastic models. In stochastic models, events do not occur at a fixed rate but with a probability inferred by the parameter value [130, 131]. These models, also called 'individual-based models', are able to take individual heterogeneity into consideration [132]. To evaluate the average behaviour in these models, modellers run many simulations and determine the mean and variance of the results [130–132].

In our field, models have demonstrated their value on several occasions. They can help generate or test hypotheses about the relationships between antibiotic use and resistance patterns [5]. For example, they can describe the impact of different treatment protocols on resistance patterns [133]. D'Agata et al. found that, among several factors possibly contributing to the emergence of resistant bacteria, delayed initiation of the antibiotic is probably the most important [134]. Models can also generate hypotheses about the most important factors to control or help test the efficacy of intervention programmes. Bonhoeffer et al. and Bergstrom et al. demonstrate that mixing strategies (i.e. the simultaneous use of different antibiotics at the population level) perform as good as or better than cycling policies to prevent the spread of resistance, while the best option to combat overall resistance is treating individuals with a combination of drugs [135, 136]. Kouyos et al. proposed and tested another strategy based on the regular switching of antibiotics in hospital wards according to the cumulative results of susceptibility testing performed on bacteria isolated from the same ward [137].

However, the strength of modelling, which allows the simulation of different situations to explore relationships between inter-dependent variables, is also its weakness. All models involve the simplification of reality and, therefore, cannot truly address the level of complexity that influences the acquisition of an antibiotic-resistant microorganism. The findings of mathematical models should always be subsequently tested in empirical studies (Table 3).

Conclusion

Choosing an appropriate study design to investigate the relationship between antibiotic exposure and its effects on resistance is critical for researchers. Up to now, many case–control and cross-sectional studies have been published. Although they provide a general understanding of the

subject, they present limitations that might impair their ability to provide further insight. Prospective cohort studies and randomised trials would help us gain a more precise understanding of the individual effects of antibiotic exposure. Moreover, multi-level studies and cluster-randomised trials might be useful for investigating the indirect effects of exposure at the community level.

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