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Joint Action
Antimicrobial Resistance and Healthcare-Associated Infections

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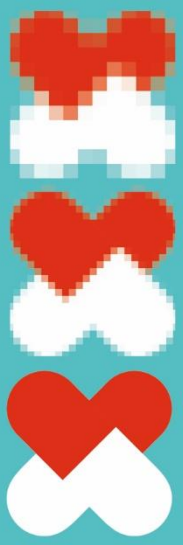
EARS-Vet MANUAL
OF METHODS AND STANDARDS



BUILDING A ONE HEALTH WORLD ♥
to reduce Antimicrobial Resistance (AMR)

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LIST OF ACRONYMS

AMC	Antimicrobial consumption
AMR	Antimicrobial resistance
AMS	Antimicrobial stewardship
AST	Antimicrobial susceptibility testing
BMD	Broth microdilution
CBP	Clinical breakpoint
DD	Disk diffusion
EARS-Net	European Antimicrobial Resistance Surveillance Network
EARS-Vet	European Antimicrobial Resistance Surveillance Network in Veterinary Medicine
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ECOFFs	Epidemiological cut-off values
EMA	European Medicines Agency
EQAS	External quality assurance system
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EU-JAMRAI	EU Joint Action on Antimicrobial Resistance and Healthcare Associated Infections
MIC	Minimum inhibitory concentration
MLST	Multi locus sequence typing
QC	Quality control
(T)ECOFF	(tentative) Epidemiological cut-off value
WGS	Whole genome sequencing

1 INTRODUCTION

1.1 Background

Antimicrobial resistance (AMR) is a major global threat to humans, animals and environmental health. AMR already represents a serious social and economic burden, and it also threatens the achievement of several of the United Nations sustainable development goals. To tackle the AMR problem, several global strategies have been developed, including the FAO Action Plan on Antimicrobial Resistance 2021–2025 and the Quadripartite's (FAO, WHO, WOAH, UNEP) joint efforts, to support integrated surveillance of AMR across multiple sectors and levels (FAO, 2021). The European Union's One Health Action Plan against AMR (European Commission, 2017) focuses on areas such as promoting the prudent use of antimicrobials and consolidating surveillance of AMR and antimicrobial consumption (AMC). This Action Plan aims to strengthen the European One Health AMR surveillance system, which is essential to identify trends and to determine how AMC and AMR are linked. Furthermore, the European Council Recommendation (2023/C 220/01) encourages Member States towards "developing integrated systems for the surveillance of AMR and AMC encompassing human health, animal health and plant health [..]".

Regulation (EU) 2016/429 on transmissible animal diseases states that "a computerised interactive information system for effective collection and management of surveillance data should be established at Union level […] and, when relevant, for emerging diseases or antimicrobial-resistant pathogens". In addition, Regulation (EU) 2019/6 on veterinary medicinal products points out the need for AMR data, and that operators and animal professionals shall have adequate knowledge of resistance to treatments, including AMR.

Links between AMC and AMR in animals and humans are investigated in the Joint Inter-Agency reports on integrated analysis of antimicrobial consumption and antimicrobial resistance in bacteria from humans and healthy food-producing animals at slaughter in the EU/EEA (JIACRA) (ECDC/EFSA/EMA, 2024). Data analysed in this report are obtained from several EU initiatives and networks. In the human sector, the European Centre for Disease Prevention and Control (ECDC) coordinates the European Antimicrobial Resistance Surveillance Network (EARS-Net), which monitors AMR in invasive bacteria isolated from blood and cerebrospinal fluid in hospitalised patients and the "food and waterborne disease network" that collects AMR data on *Salmonella enterica* and *Campylobacter* spp. isolated from human patients. In the animal sector, the European Food Safety Authority (EFSA) annually collects AMR data on healthy food-producing animals at slaughter and food thereof from all EU/EEA countries (Commission Implementing Decision (EU) 2020/1729). Despite the representative nature of the sampling and the adoption of identical antimicrobial susceptibility testing (AST) methodologies, AMR animal data target only the main food-producing animal populations (i.e. poultry and pigs / young cattle, every other year). While this existing surveillance system is valuable to assess the risk of foodborne exposure to AMR, it is of limited help to support antimicrobial stewardship (AMS) in veterinary medicine, estimate the AMR burden in the animal health sector and monitor the impact of interventions to tackle AMR. Hence,

an important gap in the European surveillance system is AMR surveillance in veterinary medicine, i.e. in bacterial pathogens of animals (Mader et al., 2021).

Of note, the European Animal Health Study Center (CEESA) financed by the veterinary pharmaceutical industry manages four programmes: European Antimicrobial Susceptibility Surveillance in Animals, VetPath, ComPath and MycoPath. However, included data are sparse, reported on an irregular basis and biased towards animals not treated with antibiotics. Hence, they are not appropriate for European surveillance purposes.

At international level, FAO launched the InFARM system in 2024, to assist countries in collecting, analysing, visualising, and effectively utilizing their AMR monitoring and surveillance data primarily from livestock, fisheries, and aquaculture, along with their associated food products (FAO, 2024). However, few countries are collecting and reporting AMR data from in bacterial pathogens of animals, and relevant guidance is needed to that end.

At national level, surveillance systems for AMR in diseased animals vary substantially between countries in terms of scope, animal species, bacterial species and antimicrobials, AST methodologies and interpretative criteria, and their reporting frequency varies (Mader et al., 2021, Mesa Varona et al., 2020, Schrijver et al., 2018). Building on existing surveillance systems and expertise, there is an urgent need to provide guidance on how surveillance of AMR in in bacterial pathogens of animals should be performed, hence improving harmonisation and comparability of the data between countries.

1.2 Objectives of the EARS-Vet network

The European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (EARS-Vet) originated from the EU Joint Action on Antimicrobial Resistance and Healthcare Associated Infections (EU-JAMRAI) over 2017–2021 and is continuing as part of EU-JAMRAI2 funded by the EU4Health programme over 2024–2027 (EU-JAMRAI website). At the time of writing this report, the EARS-Vet is composed of 38 partners from 18 countries as described in Appendix 1 (EARS-Vet network). EARS-Vet also works in close collaboration with European and international institutions (e.g. EFSA, ECDC, European Comission, FVE, FAO, WOAH, WHO).

Parallelling EARS-Net in the human sector, the EARS-Vet aims to fill in a gap on AMR surveillance in bacterial pathogens of animals in Europe. More specifically, EARS-Vet aims to report on the AMR situation, follow AMR trends and detect emerging AMR in bacterial pathogens of animals in Europe (Mader et al., 2021). EARS-Vet data can be beneficial to:

- Support AMS in veterinary medicine, e.g. to develop veterinary antimicrobial treatment guidelines adapted to the regional/country AMR epidemiological situation.
- Generate minimum inhibitory concentration (MIC) distributions as a basis for setting epidemiological cut-off values (ECOFFs) and clinical breakpoints (CBP) for the interpretation of AST results in veterinary medicine.
- Estimate the burden of AMR in animal health, similar to the assessment in human health.

- Assess the risk of transmission of resistant bacteria or resistance genes from animal origin, e.g. by direct contact with companion and food-producing animals and advise policy makers on evidence-based targeted interventions to mitigate AMR in the animal sector.
- Monitor the impact of interventions to tackle AMR in the animal sector [support the evaluation or revision of marketing authorisations of veterinary antimicrobials (in line with Regulation (EU) 2019/6)].

Hence, EARS-Vet aims to complement the existing AMR and AMC surveillance systems, by covering animal species, bacterial pathogens of animals and antibiotics of relevance to veterinary and human medicine, which are not included in EARS-Net and the EFSA monitoring (Mader et al., 2022a). In addition, following a One Health approach, EARS-Vet has been designed to allow for data to be integrated with data from other AMR and AMC monitoring systems in the animal, human and environmental sectors in Europe (e.g. as part of the JIACRA report), but also worldwide as in the FAO InFARM system.

1.3 Purpose of the EARS-Vet manual

Bringing together actors involved in national and international surveillance systems, EARS-Vet aims to act as a knowledge hub for surveillance of AMR in bacterial pathogens of animals and to provide guidance on methods and standards to be used, hence facilitating data quality, harmonisation and comparability. This EARS-Vet manual is regarded as an important resource to help in this direction, both for countries starting a new surveillance system, and for countries seeking to improve an existing system.

Inspired by other similar manuals (e.g. EARS-Net, InFARM), and leveraging the experience accumulated so far by the EARS-Vet, including a pilot study conducted in 2021–2022 (Lagrange et al., 2023), this manual provides a framework and guidelines for national surveillance systems to accurately collect, gather and organise representative data on AMR in bacterial pathogens isolated from diseased animals, furthering harmonisation. It is regarded as a dynamic resource subject to regular updates in line with evolving improvements in expertise and capacity.

2 EARS-VET SCOPE

As part of EU-JAMRAI1, a group of 26 experts (including veterinary and human medical microbiologists, and veterinary epidemiologists, affiliated with agencies, academia or ministries) from 14 European countries defined a preliminary EARS-Vet scope in 2020 (Mader et al., 2022a). This scope was defined by consensus and considering data availability among participating countries. Although the primary focus is bacterial pathogens of animals, efforts were made to define a scope that is relevant to a One Health perspective (e.g. including carbapenems which are not authorised in veterinary medicine). The scope was revised and expanded in 2024 to adapt to the needs and interests of the growing number of partners that joined EARS-Vet as part of EU-JAMRAI2.

The EARS-Vet scope is composed of relevant and feasible combinations of animals, bacteria and antimicrobial compounds to be monitored as part of the national surveillance systems for diseased animals, as shown in Table 1. Additional combinations that partners may suggest, could be considered. The EARS-Vet scope includes several animal species, covering both food-producing and companion animals. Major bacterial pathogens of animals, considered as important drivers for veterinary antimicrobial usage in Europe, were selected for each animal species. Antimicrobials were selected for specific animal-pathogen combinations, with clinical relevance, usage frequency, and resistance potential was considered for each unique pairing. The detailed lists of antimicrobials are available in Appendix 2 (EARS-Vet scope).

Future adjustments of the EARS-Vet scope will be considered depending on the epidemiology of AMR and the interests and capacities of the EARS-Vet partners. Hence, the EARS-Vet scope will be regularly updated.

Table 1. Combination of animal species, type of infection and bacterial species to be monitored in EARS-Vet

Animal species	Type of infection	Bacterial species
Cattle	digestive, mastitis, septicaemia	*Escherichia coli*
	mastitis	*Klebsiella pneumoniae* *Staphylococcus aureus* *Streptococcus dysgalactiae* *Streptococcus uberis*
	respiratory	*Mannheimia haemolytica* *Pasteurella multocida*
Cat	cutaneous, otitis	*Staphylococcus aureus*
	cutaneous, otitis, urinary	*Staphylococcus felis*
	respiratory, septicemia	*Pasteurella multocida*
	urinary	*Escherichia coli*
Chicken	arthritis, septicaemia	*Escherichia coli* *Staphylococcus aureus*
Dog	cutaneous, otitis	*Staphylococcus aureus* *Staphylococcus pseudintermedius*

	cutaneous, otitis, respiratory	*Pseudomonas aeruginosa*
	urinary	*Escherichia coli*
Goat and sheep	digestive, mastitis, septicaemia	*Escherichia coli*
	mastitis	*Staphylococcus aureus*
	respiratory	*Mannheimia haemolytica*
	respiratory, septicaemia	*Pasteurella multocida*
Horse	cutaneous	*Staphylococcus aureus*
	reproductive	*Escherichia coli*
	reproductive, respiratory	*Streptococcus equi* subsp. *zooepidemicus*
Swine	arthritis, cutaneous, septicaemia	*Staphylococcus hyicus*
	arthritis, cutaneous, respiratory, septicaemia	*Streptococcus suis*
	digestive	*Escherichia coli*
	respiratory, septicaemia	*Actinobacillus pleuropneumoniae* *Pasteurella multocida*

3 SURVEILLANCE DESIGN

3.1 Sampling strategy

European national surveillance systems of AMR in bacteria originating from diseased animals are based on analyses conducted by a single laboratory or a network of laboratories (public and/or private), with or without national central laboratories (Mader et al., 2022b). In both systems there is a national coordination centre, which supervises the implementation of the national surveillance system. When feasible, national coordination centres are designated as EARS-Vet national focal points under the responsibility of the organizations responsible for the National Action Plan (NAP) on AMR.

The level of coverage and representativeness of a surveillance system depends on the type of sampling strategy, i.e. passive or active systems. Each country should indicate the type of surveillance they conduct when uploading their data into EARS-Vet.

3.1.1 Passive surveillance

Most European national systems for AMR in bacteria originating from diseased animals are based on passive surveillance, i.e. samples from diseased animals are routinely collected and submitted to veterinary diagnostic laboratories by veterinarians and/or producers (Mader et al., 2022a). For these systems, the representativeness of AMR data needs to be assessed. As in EARS-Net, two indicators of representativeness and national geographic coverage are defined and annually estimated to evaluate the validity of the surveillance data (ECDC, 2023).

- **Population representativeness**: for each animal species, the estimated percentage of the animal population in an entire country that is under surveillance by the laboratories contributing data to EARS-Vet. This value should be considered as an indication, since the exact percentage of the population under surveillance is often difficult to assess.
  Representativeness should be assessed as semi-quantitative data:
  - high representativeness: >80% of the animal species population,
  - medium representativeness: 60-80% of the animal species population,
  - low representativeness: at most 60% of the animal species population.

  Population representativeness value to be estimated by the national focal point. For example, there is a medium representativeness if 70% of the estimated sheep population is under surveillance in a country as part of EARS-Vet.

- **Geographical coverage**: for each country across all animal species, the estimated percentage of regions, defined as NUTS-2[1] area, under surveillance by the laboratories contributing to EARS-Vet. Geographical coverage should be assessed as semi-quantitative data:
  - national or high coverage: >80% of regions are covered,
  - regional or medium coverage: 60-80% of regions are covered,
  - local or low coverage: at most 60% of regions are covered.

  Geographical coverage value to be estimated by the national focal point. For example, there is medium geographical coverage if 70% of regions within the country are covered under surveillance.

3.1.2 Active surveillance

Some other national systems conduct active surveillance where diseased animals are sampled for AMR (Mader et al. 2022b). Wherever possible, these systems should use a methodical probability sampling of bacterial pathogens of animals yielding representative and comparable data that allow statistical inferences while reducing the effect of sampling bias.

Ideally, active surveillance with randomised sampling strategies should be emphasised, with equal distribution of collected samples over the year, as it ensures population representativeness and geographical coverage while accounting for seasonality. However, the design of the surveillance plan should be adapted to the disease under study (e.g. only present in a specific group of animals, during some parts of year or in limited geographical areas). The sample size (i.e. number of isolates to be tested for susceptibility at each sampling time) should allow, within a predetermined accuracy, the calculation of the proportion of resistance to a particular antimicrobial for a given combination of bacterial species/animal populations, to follow AMR trends and to detect emerging AMR in bacterial pathogens of animals in Europe. Also, and to ensure enough statistical power, it is recommended that the sample size is reviewed by each national coordination centre considering their own situation, which also accounts for the assessment of the occurrence of resistance with sufficient accuracy (adapted from EFSA, 2019). For the sample size calculation, some insights must be considered if possible: accuracy to estimate the prevalence (report on the AMR situation) and power to detect trends (to follow AMR trends and detect emerging AMR).

[1]NUTS area (Common classification of territorial units for statistics) is a geocode standard for referencing the subdivisions of countries for statistical purposes. The NUTS regions are based on the existing national administrative subdivisions and there are three levels of NUTS defined. The current NUTS classification, valid from 1 January 2024 lists 92 regions at NUTS-1, 244 regions at NUTS-2 and 1165 regions at NUTS-3 level. https://ec.europa.eu/eurostat/web/nuts

4 LABORATORY ANALYSES

4.1 Laboratory harmonisation of antimicrobial susceptibility testing (AST) methodologies

Previous EARS-Vet study has mapped national monitoring systems for AMR in bacterial pathogens of animals (companion and food-producing) in 27 countries which indicated that they intend to build or improve their AMR systems (Mader et al., 2022b). In this context, harmonisation of AMR surveillance systems at European level is key for improving the quality of the AST data generated by laboratories, to enable inter-laboratory and inter-country data comparability.

Recently, the European Network for Optimization of Veterinary Antimicrobial Treatment (ENOVAT) identified a broad variety of methodologies and interpretive criteria used for bacterial culture, species identification, and AST of bacterial pathogens of animals throughout veterinary microbiology laboratories in Europe (Koritnik et al., 2024). Whilst selection of a particular methodology by laboratories varies and depends on many factors such as level of expertise, flexibility, level of automation and costs, progressively adopting common standardised methodologies is needed for achieving harmonised AMR surveillance. Therefore, and as stated previously, EARS-Vet aims to provide guidance on methods and standards to be used, hence facilitating data quality, harmonisation and comparability between AST results obtained by different laboratories. These methods and standard include aspects such as:

- Processing clinical specimens from the point of sample collection, bacterial culture, pathogen identification, and selection of isolates for AST. In human clinical microbiology, several manuals exist (e.g., the UK Standards for Microbiology Investigations) and could be used to guide bacteriological processing of clinical specimens (UK SMI, 2024).
- Testing of bacterial isolates from clinical samples for AST should follow European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines whenever applicable. Regarding AST techniques, both broth microdilution (BMD) and disk diffusion (DD) are accepted, regardless of the AST instrument(s) used in the laboratory.
- It is acknowledged that many veterinary diagnostic laboratories use commercial systems, such as the VITEK®2 automated instrument, that test a limited range of dilutions. This results in incomplete data that cannot be used for all EARS-Vet purposes. Where this is the case, it may be necessary to retest selected isolates with a MIC BMD method including dilution range that allow determination of a (tentative) epidemiological cut-off value [(T)ECOFF] and submission of complete data to the EARS-Vet system.
- AST reporting procedure: reporting to the client and to EARS-Vet should be independent and tailored to the therapeutic and surveillance aims, respectively.
- Resistance mechanisms that should be included for routine testing for AMR surveillance for human bacterial isolates are described in EUCAST guidelines (EUCAST, 2017). Resistance mechanisms that are relevant to veterinary clinical isolates include the following phenotypes:
    - Carbapenemase production by Enterobacterales

- Extended-spectrum β-lactamase (ESBL) production by Enterobacterales
- Acquired AmpC β-lactamase production by Enterobacterales
- Polymyxin resistance in Gram-negative bacilli
- Carbapenemase production by *P. aeruginosa*
- Methicillin resistance by *Staphylococcus aureus* and *S. pseudintermedius*
- Vancomycin resistance by *Staphylococcus aureus*
- Inducible clindamycin resistance in staphylococci and streptococci (see EUCAST breakpoint tables)

- As per EUCAST guidelines, where a resistant phenotype is expected (previously 'intrinsic resistance'), either no result should be reported for that antibiotic agent, or the result should be reported as resistant without testing.
- Good AMS practice involves selective or cascade reporting. It is recommended that results for only European Medicines Agency (EMA) categories C and D antibiotics should be reported where the organism being tested is susceptible to one or more of these agents. However, laboratories need to ensure that suppressed results are available for AMR surveillance and for reporting in the event that a justifiable clinical reason is provided for releasing the result. For example, consider reversing suppression of results after the clinical report has been issued.

4.1.1 Bacterial isolate selection for AST

Bacterial culture interpretation is a valuable and complex skill developed through specialist training which helps the clinical microbiologist to determine which bacterial isolates are likely to be associated with an infection. Amongst many aspects of the bacterial culture interpretation, the clinical and paraclinical information provided as part of clinical history will help the laboratory in this interpretation process. Additionally, bacterial identification at species level is one of the key aspects required for interpretation of its likely clinical significance, determining if AST is indicated and which isolates are selected for this investigation. Selecting isolates from normally sterile sites is generally straightforward when the sample is collected appropriately, as bacterial isolates obtained in pure culture are likely to be clinically significant. However, great care needs to be taken for isolate selection for AST as regards bacterial cultures from normally non-sterile sites (e.g. wound infections, upper respiratory or reproductive tract) because polymicrobial cultures are usually yielded. Ideally, the decision of which isolates to be selected for AST, should also be supported by cytology findings demonstrating inflammation and the presence of bacterial morphologies consistent with that of the identified organisms via bacterial culture. The main risk lies with the fact that many bacterial isolates form part of the commensal microbiota and may be intrinsically resistant to antibiotics, therefore unnecessary AST may result in the clinician using a higher tier antimicrobial for an isolate which could be part of normal bacterial microbiota.

4.1.2 Laboratory methods

Careful laboratory practices including good quality control (QC) procedures are essential in ensuring results generated are reliable and provide accurate surveillance data for inclusion in EARS-Vet. EUCAST methods should be followed with strict adherence to the standard operating and QC procedures specified, whether BMD or DD methods are employed. Where commercial automated systems such as the VITEK® 2 instrument are used, manufacturer's guidelines should be followed. A laboratory quality management system should be in place with quality assurance procedures to ensure timely detection of errors or problems. Ideally, laboratories should be accredited by an approved national body and participate regularly in proficiency testing run by an approved independent entity. A brief description of BMD and DD methods follows with detailed descriptions provided in Appendix 3 (Description BMD-DD methods).

4.2 Broth microdilution method (BMD)

The reference strains proposed to be used for AST against bacteria targeted within the scope of EU-JAMRAI2 are listed in Appendix 3, as well as in appropriate EUCAST guidelines.

MIC plates (i.e., BMD panels) covering the majority of bacteria-antibiotic combinations of interest to EARS-Vet were designed within EU-JAMRAI2. Detailed information is provided in Appendix 3.

Detaied description of the BMD methodology is provided in Appendix 3. The reader is highly advised to refer to the EUCAST website regularly, to ensure that the latest updates of methodological aspects are taken into consideration when performing MIC determination via BMD.

4.3 Disk diffusion (DD) method

The DD method should be conducted based on EUCAST guidelines. QC strains for DD method are the same as those listed in Appendix 3 for BMD.

Details of the DD method are provided in Appendix 3 and are in line with EUCAST guidelines. However, the reader is advised to refer to the EUCAST website regularly to ensure that all methods used are in line with any published updates.

5 AST DATA COLLECTION AND ANALYSIS

For effective cross-country comparisons and analysis of AMR trends in bacterial pathogens of animals, the EARS-Vet aims to collect harmonised AST data from all participating partners. This section provides an overview of the entire EARS-Vet surveillance process, from initial data generation to final joint reporting. The process is divided into four main components.

5.1 Data sources

The collected results originate from both passive and active surveillances conducted across various European countries and are held by EARS-Vet partners. The data included are at least those refered in the scope (see section 2), but it is also possible for partners to include additional data. Historical data are accepted; however, moving forward, it is recommended that future results adhere as closely as possible to the guidelines outlined in this manual (see section 4.2 and section 4.3).

The EARS-Vet encourages prioritising clinical cases over incidental or screening isolates in submissions, though partners can include all isolates with AST results when clinical data are unavailable. To allow the results to be re-interpreted for the purposes of harmonisation (section 5.3.3), only the results of the tests carried out by BMD or DD techniques will be accepted, regardless of the AST instrument used.

5.2 Data requirements

The data collection template can be found in Appendix 4 (Data collection template) and it is summarized in Table 3. The template presents the data dictionary, detailing all mandatory and optional metadata fields. Each field is accompanied by a definition, potential values, and the expected data format, ensuring consistency and clarity in data submission.

For each antibiotic tested per animal-bacteria combination, the assay technique of AST (BDM or DD) must be specified, along with the raw value (i.e. MIC or disk diameter value) obtained. The AST standard used is mandatory, as is the antibacterial concentration of disks when DD is employed. While some fields are optional, they are highly recommended and should be included when available. The specimen type will be used to derive the infection type for those partners unable to inform infection type directly.

These data collection requirements will be updated by consensus within the EARS-Vet whenever needed (as a minimum every 5 years). Suggestions for adjustments are welcomed and will be considered when updating the data requirements.

Table 3. Data requirements

	Name of the variable	Mandatory	Definition

General	**Isolate ID**	y	Unique isolate identification code. An isolate is a single strain of microorganism isolated from a sample (characterised by a combination of an animal/bacteria/specimen type/sampling date)
	Country of animal	n	Country in which the animal lives, ISO2-code
	Partner	y	Identifier of the EARS-Vet partner
	Year of testing	y	Year the isolate was tested
Isolate	**Animal species**	y	Animal species the isolate is originating from
	Age category	n	Age category of the animal from which the sample originates
	Infection type	n	Type of infection affecting the animal from which the isolate originates
	Specimen type	n	Specimen types
	Bacterial species	y	Isolated bacterial species
	Antimicrobial agent	y	Antimicrobial agent being tested for
	Disk diameter or MIC value	y	Value of the inhibition zone diameter (in mm) or the minimum inhibitory concentration (MIC) (in mg/L or µg/mL)
	Virulent strain	n	If a test was carried out to look for a virulent profile (ETEC), was the result positive or negative?
	ESBL profile	n	If a test was carried out for an ESBL profile, was the result positive or negative?
	AmpC phenotype	n	If a test was carried out to look for an AmpC profile, was the result positive or negative?
	PCR mecA	n	If a PCR test was carried out to characterise a methicillin-resistant profile by looking for the mecA gene, was the result positive or negative?
	PCR mecC	n	If a PCR test was carried out to characterise a methicillin-resistant profile by looking for the mecC gene, was the result positive or negative?
Test	**AST technique**	y	Technique used for antimicrobial susceptibility testing (AST)
	AST instrument	n	Instrument used for antimicrobial susceptibility testing (AST)
	AST standard	y	Standard used for AST procedure
	AST standard_other	n	In case **AST standard**=other, please specify here which standard is being used
	Disk content	n	Concentration of the antimicrobial drug in the disk
	Virulence testing technique	n	Technique used for virulence testing
	Virulence testing technique_other	n	In case **Virulence testing technique**=other, please specify here which technique is being used
	ESBL confirmation technique	n	Technique used for confirmation of ESBL profile

	ESBL confirmation technique_other	n	In case **ESBL confirmation technique**=other, please specify here which technique is being used
	AmpC confirmation technique	n	Technique used for confirmation of AmpC profile
	AmpC confirmation technique_other	n	In case **AmpC confirmation technique**=other, please specify here which technique is being used

Data on population representativeness, geographical coverage will be collected via a separate annual survey since these data are collected at partner level (rather than at the isolate level).

5.3 Data submission and management

The terms governing the sharing of AST data are set out in the EARS-Vet data sharing agreement (Appendix 5). Signing this document is mandatory for all partners wishing to join the EARS-Vet and contribute with AST data. The technical tutorial for data submission can be found in Appendix 6.

5.3.1 Data preparation

Duplicates managements: when multiple isolates of the same bacterial species originate from the same companion animal or the same farm over the same year (repeats over time) or on the same day, it was decided that all isolates should be kept. This decision was made for both technical reasons (since not all partners are able to identify repeats in their datasets), and scientific reasons (i.e., discarding repeats in the isolates could lead to remove important data, such as multi-drug resistant isolates). In addition, it is believed that repeat isolates represent a minor proportion of EARS-Vet data and have a minor impact on resistance proportions. However, this might differ between partners and will be explored in future dedicated studies.

Hence, partners must remove duplicates in only two cases: i) when a data entry error has led to the same isolate being recorded twice, and ii) in the rare cases where two isolates from the same sample have exactly the same SIR profile (duplicates due to the way the tests were carried out in the laboratory). At this stage, duplicate management should be performed by the data provider prior to data submission.

Data formats: to submit data to EARS-Vet, the data must be in the format described in the data collection template (Appendix 4). Partners must therefore format their data, either using their own resources or using a feature of the WHONET software developed for this purpose.

WHONET is a free Windows-based microbiology laboratory database software developed by the WHO Collaborating Centre for Surveillance of Antimicrobial Resistance, and it is designed for effective processing and analysis of AMR data. It is used by several other surveillance programmes, including EARS-Net and FAO InFARM system. The software streamlines routine data entry and enables export of data in the EARS-Vet expected format. For additional information, a tutorial document is available on the respective training centre (WHONET Training Center).

5.3.2 Data submission

Every year by June, partners who have signed the data sharing agreement send files in a harmonised format, with consolidated data from the previous year. To ensure confidentiality, any personal information related to animals or farms is excluded from the submitted data (i.e. pseudo-anonymization prior to submission).

EARS-Vet partners are provided with login details to submit their data on a secured server, preferably in .txt format, using tab separators. Data are automatically checked for correct formatting, completeness and consistency. In the event of non-compliance, a message is sent to the data providers to correct their data. When the data meet the requirements, they are integrated into the EARS-Vet database hosted by ANSES. A summary report describing the included data is sent to the submitting institution. This allows the submitting institution to check that no data have been lost during the submission process.

5.3.3 AST Data interpretation

The AST raw results (i.e. MIC values or DD diameters) are interpreted by ANSES using available interpretation criteria (table under construction). Where available, the (T)ECOFFs thresholds defined by EUCAST are used. In the absence of these thresholds, the CBP defined by CLSI are applied. The breakpoints used as a priority are those for the correct combination of animal/bacteria/antibiotic/infection type, but this can be adpated in the event of unavailability. For tests performed by DD, interpretation will be performed using the standard used to perform the test.

5.4 Data analysis and reporting of results

The EARS-Vet data analysis follows a structured approach to comprehensively assess AMR resistance trends. Following Arieti et al. (2020), trends and maps are displayed for selected combinations of bacterial species and antibiotic categories, with a minimum threshold of 30 isolates per year, animal species/categories, and country. To account for the diversity of antibiotics tested, data analyses group antibiotic agents with similar resistance mechanisms into categories, as proposed by Mader et al, 2022a. Resistance percentages are thus calculated at multiple levels: for individual antibiotics, antibiotic classes, and multidrug resistance.

Analysis is conducted using appropriate statistical software (e.g. R). The results of the analysis are synthesised and presented in an annual report, providing stakeholders with a detailed overview of AMR patterns in veterinary medicine across participating countries.

An interactive dashboard will also be developed for users to select and visualise data of interest.

6 WHOLE GENOME SEQUENCING (WGS)

Whole genome sequencing will be used for characterization of specific bacterial pathogens of animals with selected phenotypes as part of EU-JAMRAI2 EARS-Vet. A subset of strains will be sequenced and analysed bioinformatically. Additionally, as the use of WGS has been implemented in several partner institutions, further data will be collected for phenotypes of interest and/or to investigate specific pathogens species/types.

6.1 Sample and strain selection for WGS

The selection of phenotypes of interest is based on the EARS-Vet scope agreed upon (Appendix 2) and finally decided after a scoring exercise performed by the partners actively participating in subtask 8.2.3 (i.e. WGS analyses of phenotypes). Furthermore, a survey among all partners participating in these subtasks has been used to assess the availability and numbers of existing and prospective isolates with the highest scoring phenotypes, so that a decision regarding the criteria for inclusion and exclusion for WGS was established. The prioritized list of phenotypes (equal or above score 20) include:

- ESBL/AmpC-producing *E. coli* and *Klebsiella*,
- carbapenemase-producing and colistin-resistant *E. coli* and *Klebsiella* from all relevant host species,
- methicillin-resistant *Staphylococcus aureus* (MRSA/MRSP) (only pathogenic) from cattle, chicken and pets and vancomycin- or linezolid-resistant *Staphylococcus aureus*.

It is expected to perform WGS in years 2 and 3 in the sequencing partner institutions which have received funding for this task, namely IZSLT for the Italian isolates, ANSES for the French isolates, and NVI and SSI will share the isolates from the remaining partners. Partners providing isolates for sequencing will be invited to send their isolates to these laboratories to uniformise/harmonise the entire procedure. The providing laboratories will use their own material transfer agreement forms for isolate sharing with the sequencing partner. In some cases, sharing of DNA might be accepted, if the quality of the received DNA is acceptable and if the extraction method used aligns with those described in section 6.2.1. For the labs that have already performed WGS, there will be opportunities to include their sequencing results in the analyses, provided that these are of sufficient quality (see section 6.4).

6.2 DNA extraction and library preparation

6.2.1 DNA extraction

DNA shall be extracted from a pure culture of the selected isolates cultured on non-selective media (agar or broth). DNA extraction may be performed using in-house methods or commercially available extraction kits (e.g. ThermoFisher or Qiagen DNA extraction kits for blood and tissue) or

high throughput DNA extraction equipment (as for example the MagnaPure or QIAsymphony SP systems) as long as the method assures extraction of total genomic DNA including chromosomal and extra-chromosomal DNA. DNA extraction should be done according to the manufacturer's instructions. Any deviation from the provided protocol should be described and justified.

It is not recommended to use ethanol precipitation methods due to poor performance for extracting plasmid DNA, which will influence the results regarding AMR genes carried on plasmids.

The DNA concentration should be estimated using fluorometric assay kits (e.g. Quant-iT dsDNA BR) with fluorometric reading (in e.g. a Qubit or FLUOstar Omega) and/or spectrophotometric systems. If the concentration measured is below the minimum concentration required for the library preparation, it is necessary to repeat the extraction procedure. If the concentration is higher, DNA can be diluted to the correct concentration using an adequate buffer without EDTA and/or sodium azide (or nuclease-free water). The concentration should be confirmed by new measurements after dilution.

DNA purity shall be determined using a spectrophotometric system to assess the A260/A280 (1.8 - 2.0) and A260/A230 (>1) ratios.

6.2.2 Library preparation

Library preparation is necessary to fragment and tag the amplicons that will be sequenced by the sequencing equipment.

There are several methods for library preparation, depending on the chosen DNA sequencing platform. The most common method for paired-end sequencing in the Illumina platforms is to use library preparation kits such as the Illumina DNA prep or Nextera XT library preparation kit (Illumina). For detailed and updated information regarding DNA preparation for Illumina, consult the reference guide on the Illumina webpage: Illumina DNA Prep Reference Guide (1000000025416) and/or the Nextera Library prep reference guide: Nextera DNA Library Prep Reference Guide (15027987) (illumina.com)

The multiplexed libraries (also denominated as pool) should be quantified using either fluorometric assay kits (e.g. Qubit 1X dsDNA High Sensitivity kit) with fluorometric reading (e.g. a Qubit 4 Fluorometer) and fragment sizes analysed on a TapeStation 4200 (Agilent), before final library dilution and denaturation.

6.3 Sequencing

For the general purpose of isolate characterisation as part of EARS-Vet, short-read sequencing on Illumina platforms (MiSeq, HiSeq or NextSeq) is preferred. Long-read sequences might be used for specific purposes and/or might be accepted as pre-existing data from partner institutions if the QC criteria are fulfilled. Throughout all these processes, good record keeping and tracking of sample

progress should be employed to allow construction of a full audit trail, for example using a LIMS system or equivalent.

As an example, the sequencing can be performed on the NextSeq 550 platform (Illumina) using a v. 2.5 Mid Output 300-cycle Sequencing Reagent kit to obtain 2 x 150bp paired-end reads. After sequencing, extract the raw data files as fastq which include also quality values (Phred scores).

6.4 Quality control (QC) of sequencing data

QC of sequencing data must be performed before initiating any bioinformatic analyses. Poor data quality may result in errors, such as lack of detection, failure in detecting contaminants and or other issues leading to misinterpretation. Quality of data can be visualised within cloud-based solutions as is the case of Illumina sequence hub or analysed using online tools such as FastQC and MultiQC, and/or using in-house pipelines for QC. Adapter removal should be performed, and, if necessary, poor quality data should be removed using software such as Trimmomatic or TrimGalore.

It is important to assess the data in relation to possible contaminants. For instance, taxonomic classification software can be used to identify potential contaminants. One example is the k-mer based tool KRAKEN2 which can be used to quantify the number of reads assigned to other species than the target species. The percentage of reads assigned to other species should be limited (for example less than 5%). ID checks can also be confirmed with online tools such as KmerFinder or rMLST.

QC pipelines including the major parameters to be analysed facilitate the QC process providing valuable information to the Core facility and users of the sequencing data.

An example of an in-house QC pipeline is the QC of the raw fastq via a modified pipeline for WGS analysis, bifrost (https://github.com/ssi-dk/bifrost), based on the SKESA assembler, which includes comparison of identified species, or genome size with the provided, the major parameters for QC obtained, conclusion in relation to these as pass or warnings to the laboratory, and need for follow-up based on failed QC parameters.

If the QC requirements are not fulfilled (Table 2), the DNA should be re-sequenced. If there is indication of contamination, DNA should be re-extracted from a pure culture to avoid contamination. The species of the pure culture should be confirmed before a new DNA extraction is initiated. See below a schematic representation of workflow regarding QC and bioinformatic analysis (Figure 1).

Table 2. Minimum QC requirements – raw sequencing reads

QC parameter	Requirement
Per-base Phred-score	≥ 20
Number of reads	As high as possible

Coverage (C), calculated as read length (L) divided by genome size (G) multiplied by number of reads (N): C = N * (L/G)	>30
Contamination	< 5% of reads classified as another species than the species sequenced
Average read length	Identical to expected read length from the sequencing platform used. Deviations indicate sequencing error.

In addition, the GC content of the sequences should be evaluated. If this varies from the expected GC content of the species sequenced, it may be an indication of contamination.

6.4.1 Bioinformatic analyses and tools (including QC)

The tools used for analyses, including version of tools and databases should be recorded by the partner performing the analyses (see section 6.7) in order to ensure reproducibility of the results. If analyses are run on the command-line, the specific code used should also be recorded.

6.4.2 Genome assembly

De novo assembly using Shovill, SPAdes, Unicycler, SKESA or similar. The quality of the assemblies should be assessed, using for example Quast.

Minimum QC requirements – assemblies:

- Total length of assembly – within ±20% of expected genome size for the species
- Number of contigs should be < 500
- N50 indicates the size of contigs in general. Suggest using > 30 000 bp (Bortolaia et al., 2020)

6.4.3 Multi locus sequence typing (MLST)

For species where an MLST schema is available, the sequence type (ST) can be determined using the command-line tool mlst, which is linked to the PubMLST typing schemes. MLST can also be determined using online tools such as MLST.

6.4.4 Resistance genes /point mutation finder tools

Multiple tools are available for identification of AMR-encoding genes and/or point mutations resulting in resistant phenotypes. The most used are ResFinder, AMRFinder and CARD.

For example, the EURL-AR recommends running ResFinder tool on assemblies, using the following settings:

- %ID: 90%
- Minimum length 60%

6.4.5 Plasmid finder tools and plasmid characterization

AMR genes are commonly associated with mobile genetic elements, such as plasmids. The presence of plasmids and/or other mobile genetic elements can be assessed using, for example, PlasmidFinder, mobsuite or MGEFinder . Knowledge regarding MGE-AMR gene links can be important to elucidate the molecular epidemiology and trace dissemination of AMR in bacterial populations. These analyses can be performed if more in-depth characterisation of sequenced isolates is required.

6.4.6 Phylogenetic and/or clustering analyses

Core-genome MLST (cgMLST) schemes are available for some bacterial species, which can be run with the chewBBACA suite and utilised to evaluate clustering of included isolates.

SNP-based approaches can also be used if the isolates are more closely related, i.e. snippy (performed on reads using a reference genome) or ParSNP (performed on assemblies, reference free).

6.4.7 Serotyping, fimbria-based typing or other typing tools

Other typing tools are also available for characterization of isolates, such as determination of serotypes, fimH types and presence of virulence genes but this manual does not include a detailed description of these tools.

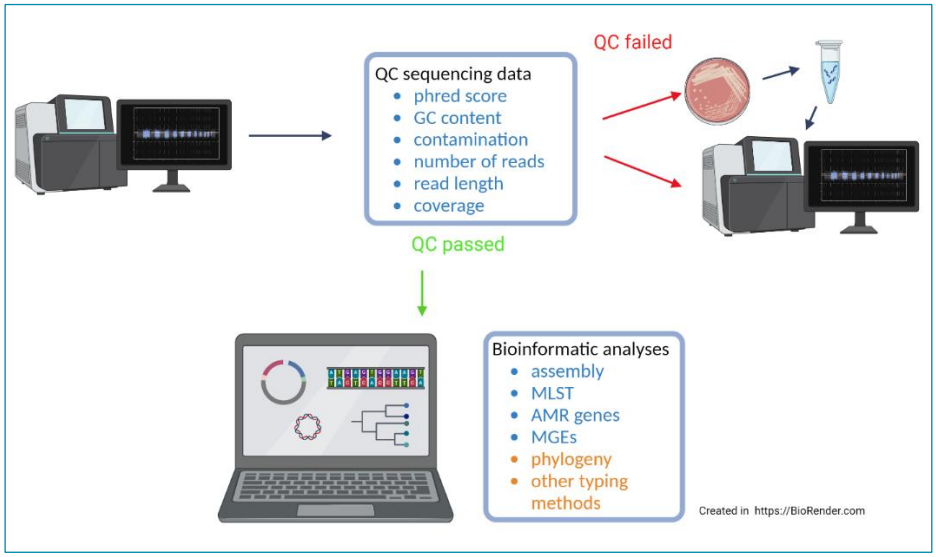

Figure 1: Illustration of workflow for QC and bioinformatic analysis of sequencing data. Blue points indicate mandatory analyses that should be performed on all isolates sequenced, while orange points indicate optional analyses that can be performed if indicated.

6.5 Interpretation of results

6.5.1 Interpretation of genome data

The genomic data analyses should be interpreted only if the data are of sufficient quality and considering the limitations of the tools utilised in the analysis. For example, it is not possible to detect unknown genes or mutations that are not included in the databases used. There is also variation between versions of the databases. When interpreting genomic results, it is also necessary to be aware of what is expected in a given species as it might be a means to detect errors or issues relevant to the data. Species specific tools such as MLST and point mutation finders depend on the species identification correctness. For interpreting resistance mechanisms, it might be necessary to be aware of the impact of these on expected phenotypes since some genes might change the phenotype for a given antimicrobial more than others of the same class, or the specificity for given antimicrobials within the target class might vary.

It is also important to be aware of genes and mutations that are always or very frequently present in certain species or serovars and therefore can be considered intrinsic, while others are acquired.

6.5.2 Resistance/Virulence phenotype predictions

In some of the resistance gene and point mutation tools, there are added predictions related to the expected phenotypes for these, ex ResFinder 4.0. However, despite the abundance of information, the prediction of AMR phenotypes is very complex and might be incomplete or erroneus due to:

- intrinsic resistances that are not predicted
- acquired genes that confer resistance to additional antimicrobials not included in the prediction
- resistance genes or mutations that do not confer resistance phenotype in some strains
- changes in phenotypes due to compensatory mutations and/or interaction with other mechanisms or efflux
- resistance due to changes in regulatory systems and permeability that are not detected
- unknown resistance genes or mutations which are not yet in databases and therefore not detected, etc.

Also, it is important to consider that most databases are mainly based on the most frequent bacterial species isolated from humans and might still be under development and relatively poor when analysing certain non-zoonotic veterinary pathogens. Similarly, the prediction tools include representatives from the major antimicrobial classes used in human medicine but are less complete in relation to antimicrobial compounds used exclusively in veterinary medicine. On the positive side, WGS predictions are accurate for major pathogens and the analysis can be performed in a timely

manner; results of resistance, virulence and high resolution typing can be shared and combined with epidemiological data for efficient tracing of outbreaks and spread of certain clones and/or resistance carrying mobile genetic elements across different laboratories, countries and sectors, replacing older molecular methods.

6.6 External quality assurance systems (EQAS)

Even though these EQAS are not directly financed by the JAMRAI2 funding, it is advisable for the participants performing sequencing to participate in EQA proficiency tests or ring trials to evaluate the whole process or parts of it, starting from the isolate, DNA or sequences. The partners involved in surveillance for EFSA are participating in EQAS by the EURL-AR which include WGS components in some of the trials.

Examples of EQAS: DTU Genomic PT, EURL-Campylobacter PT on WGS, EURL-Salmonella PT Typing and Cluster analysis, EURL-*E. coli* PT pathogenic *E. col*i, EQA scheme for typing of STEC.

6.7 Data (and metadata) storage and data sharing

Whichever solution is chosen from the listed options, the location for the long-term storage of the data should be considered. Although data can be uploaded to the public nucleotide archives (e.g. EMBL-EBI (https://www.ebi.ac.uk/ena/submit/sra/#home) or NCBI (https://www.ncbi.nlm.nih.gov/sra/docs/submitportal/), it is likely that local storage of the files will be necessary. The amount of storage space required will be in the order of several terabytes per partner. A resilient storage system recommended by local IT should be available to store a large amount of data. Raw sequence data must be accompanied by minimum metadata parameters as for example metadata of the isolate: year of testing; geographical origin; source; sample type; expected species. The metadata should be aligned with EARS-Vet database requirements for direct correspondence. Additionally information should be reserved for the following: storage location; date of DNA extraction; kit used; DNA concentration; details on library preparation protocol; date of preparation; kit used; DNA concentration of each input library; layout of the microtiter plate; normalisation and dilution approaches; sequencing platform and sequencing run: platform name; sequencing run number; sequencing start date; sequencing end date; sequencing yield; raw data QC: average read length; coverage; number of reads.

Similarly, assemblies and analysis results should be stored and, in either case, reserved information about the processing, such as tools and databases versions used, date of run, any interpretation guidelines used, etc.

6.8 Major uses, advantages and pitfalls regarding WGS results in surveillance

There are major advantages to the use of WGS in surveillance, which lead to the trend in increased use of the technology. Besides the technical aspects and cost related issues, the tools for veterinary pathogens need further development since these are not as developed as human-related tools. Similarly, the prediction tools will still be under development due to existing gaps. However, undeniably WGS is well-entrenched and offers major advantages regarding data comparability (between labs and even sectors), data sharing and harmonisation of methodology. Moreover, WGS data availability will allow flexibility to use and compare different tools for analysis, both for characterization and high-resolution typing, cluster analysis, as well as for detection of virulence and resistance mechanisms and prediction of phenotypes. In the future, when more data are available, the integration of WGS data in EARS-Vet will be even more helpful providing more complete and rapid responses that will enhance the prediction of trends, design and decision making for interventions and treatment guidance.

7 LIMITATIONS

There are some limitations associated with the EARS-Vet surveillance system. The data to be collected in many countries may be incomplete and may not be representative of all diseased animals in a specific region. The data may be biased as they may include isolates from both untreated and previously treated cases; in most cases, this information is not available and not provided by the test laboratories to the surveillance system of EARS-Vet partners.

Not all diagnostic laboratories, in a particular region or country, participate in EARS-Vet and the client base of laboratories is likely to differ, resulting in lack of data representativeness. A further difficulty, again due to poor or incomplete sample history and inability to decide on clinical relevance, may be the inclusion of test results of isolates that are clinically insignificant.

Testing methods employed in the participating laboratories are highly diverse and present difficulties for harmonisation of results. However, this will be addressed by having laboratories submit raw MIC and DD zone diameter data and by retesting a subset of isolates from the laboratories using the customised EARS-Vet MIC plates. Thus, completely harmonised data will be available for at least a proportion of isolates.

The lack of (T)ECOFFs for interpretation of data from many animal/pathogen combinations is a limitation that will be addressed over time as these are generated as part of the EARS-Vet and other projects.

The sustainability of EARS-Vet beyond EU-JAMRAI2 has not been guaranteed at the time of publication of this EARS-Vet manual version 1.0. Thus, the analysis of data and publication of a report with the results need further discussions to reach an agreement.

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EU-JAMRAI

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