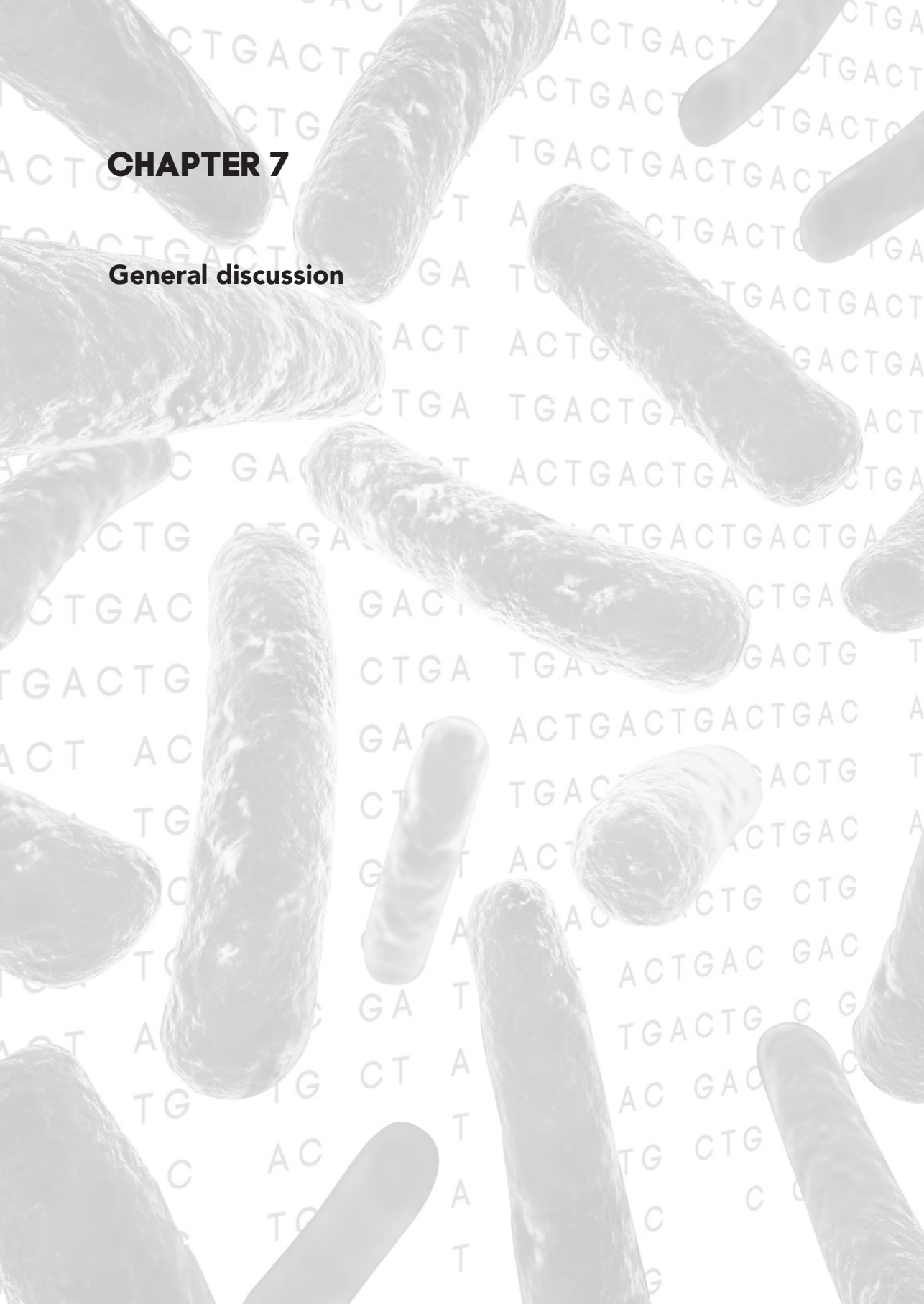

CHAPTER 7

General discussion

7.1 The continuing problem of antimicrobial resistance

Antimicrobial resistance among *Enterobacteriaceae* has progressively increased and has accelerated within the past 15 years. The spread of resistance is unlikely to stop and may compromise the value of established antimicrobial agents. The increase in resistance is mainly due to the spread of clones carrying genes encoding for extended-spectrum ß-lactamases or carbapenemases.[1-3] A report from the European Center for Disease Prevention and Control published in 2009 estimated that approximately 400,000 infections in Europe were associated with resistant strains, resulting in 25,000 extra deaths and 2.5 million extra hospital days.[4] The Dutch surveillance report NETHMAP 2013 indicates that 6% of the clinical *Klebsiella pneumoniae* and 8% of the *Escherichia coli* isolates recovered from unselected hospital departments are highly resistant microorganisms.[5] Increasing resistance rates to 3rd generation cephalosporins are reported from 3.5% in 2008 to 5.7% in 2012 for *E. coli* and from 4.0% to 7.5% for *K. pneumoniae* respectively. At present, cefuroxime and gentamicin are still used as empirical therapy in case of sepsis in the Leiden University Medical Center (LUMC). Given the rise of multidrug resistance and the mechanisms involved in *Enterobacteriaceae* in our hospital setting, it remains to be seen whether this will continue to be the appropriate choice. Despite the recognized need for new antimicrobials for clinical use, the reality is that only two new classes of antibiotics, oxazolidinone and lipopeptide, active against Gram-positive bacteria have been brought to market in the past 14 years.[6] The Pew Charitable Trusts is a large independent non-profit, non-governmental organization founded in 1948, that has assessed antibiotics currently in clinical development, which is available online and updated regularly (http://www.pewhealth.org/other-resource/antibiotics-currently-in-clinical-development-85899541594).However, given the inevitability that some in development will fail to obtain final approval, it is clear that there are too few drugs in development to meet current and anticipated patient needs.

The aim of the research described in this thesis was to obtain more insight into the four principal mechanisms of antimicrobial resistance in multidrug resistant *Enterobacteriaceae*, recovered at the LUMC, the Netherlands. This discussion will try to define some useful future strategies, based upon the results of this thesis and the other recent insights that will first be briefly summarized.

7.2 Necessity of rapid molecular detection

Antibiotic resistance genes were present among clinical isolates at very low levels prior to the introduction of antibiotics, and it is largely the selective pressure of antibiotic use, not only in humans but also in pets and livestock, which has caused the rise. The increasing mobility of people across the globe has further contributed to the spread of antimicrobial resistance. The rise in antimicrobial resistance relates not only to the number of individuals infected or colonized with antimicrobial resistant *Enterobacteriaceae*, but also to the diversity of underlying resistance mechanisms, which will continue to increase in the years ahead. In multidrug resistant strains of today, it is common that all four of principal mechanisms play a role in resistance, with strains having multiple determinants affecting susceptibility to each antimicrobial agent.

As shown in all chapters of this thesis, molecular characterization of antimicrobial resistance mechanisms illustrates the heterogeneity of antimicrobial resistance mechanisms involved in multidrug resistant *Enterobacteriaceae*. Besides important transferable extended-spectrum ß-lactamases, CTX-M, SHV, TEM, OXA, plasmid mediated AmpC ß-lactamases, carbapenemases, other enzymes such as the plasmid-mediated quinolone resistance Qnr and AAC-6'-Ib-cr should be tested for *Enterobacteriaceae*. As described in chapter 2, 3, and 4 molecular characterization revealed that chromosomally-encoded resistance traits such as mutations in the GyrA, ParC, ParE, chromosomal efflux activity, c-AmpC with its promoter/attenuator region are of importance as well. Although multiplex PCR and sequence analysis, qRT-PCR and microarrays are well established methods, as used in various chapters of this thesis, their use is insufficient to investigate the numerous genes involved. In the current era of multidrug resistance, molecular tests need to be rapid and highly multiplexed and multifaceted in a single assay. Therefore, improved diagnostic approaches for the rapid and adequate detection of antimicrobial resistance, epidemiological markers and virulence characteristics are essential. Besides early and sensitive detection of resistance mechanisms by non-phenotypic tests, preventive measures and active surveillance will become more important in the near future.

7.3 Implications for future strategies

7.3.1 Potential for whole genome sequencing

Microbiological laboratories play a central role in the combat against antimicrobial resistance trends by rapid and proper recognition of multidrug resistant *Enterobacteriaceae*. The use of phenotypic tests, PCR-based techniques and microarrays has all demonstrated their utility to detect resistance. To obtain a better understanding of the current molecular epidemiology and characteristics of multidrug resistant *Enterobacteriaceae* more information is required, e.g. the type of antimicrobial resistance mechanism, presence or absence of resistance genes and specific mutations conferring resistance.

With the emergence of the new tools of whole genome sequencing (WGS), it allows to sequence full bacterial genomes in an efficient way. WGS enables an integrated approach to study the complete genome of a bacterial isolate, which can be determined within hours, providing identification to the species, its susceptibility to antibiotics, its virulence characteristics and epidemiological markers, as described in chapter 6. This technique has become fast, making WGS compatible with the routine microbiology workflow.[7,8] A sample preparation method for rapid single colony WGS of different bacterial species has been validated for clinical use.[9] It is therefore to be considered likely that applications of WGS will play an important role in the approach to antimicrobial resistance and the following paragraphs will discuss some relevant topics for future research, related to this development.

7.3.2 Understanding and predicting antimicrobial resistance

First attempts to predict antimicrobial susceptibilities using WGS data in different species, such as *Staphylococcus aureus*,[10] *Mycobacterium tuberculosis* [11] as well as for *E. coli* and *K. pneumoniae* [12] have been reported recently with promising results. Despite rapid advances in this field and major advantages of this technique, WGS for the detection of antimicrobial resistance is still not yet widely adopted by clinical microbiology laboratories. The WGS approach in the absence of any phenotypic support for the functionality of a resistance gene or mutation could lead to major errors (reported as resistant and actually susceptible) and preclude the use of potentially useful therapies. Conversely, the absence of an identifiable gene or mutation sequence does not guarantee susceptibility. To be used confidentially in clinical practice, reliable genotypic prediction of the antimicrobial resistance

phenotype has to be demonstrated to the same standards as any new phenotypic method, using large diverse sets of unrelated isolates. Comprehensively validated genotypic prediction of antimicrobial resistance, ready for implementation in clinical practice will require multiple large studies.

In fact, it is likely that phenotypic methods will continue to be used, at least for the near future, to screen microbial isolates for unrecognized resistance patterns, and, thus, their mechanisms of resistance, before gene-level inquiry is pursued. However, the need for routine phenotyping will diminish as laboratories increasingly use WGS data, combined with phenotypic data, to elucidate the contribution of all underlying genetics in resistance. Further investigation is required to determine to what extent it will be possible to rely on genotype alone. The need for phenotypic verification of the genotype might be partly circumvented by WGS transcriptomics using RNA-Seq, thus, providing direct evidence of functional resistance rather than using gene identification.[13]

Although WGS is not suitable for resistance prediction in clinical practice yet, sequencing the whole genome provides full characterization of a bacterial strain, providing all genetic information on species identification, sequence type and virulence determination in a single test. WGS as a single platform for extracting all the information required is an attractive alternative in the routine diagnostic setting.

7.3.3 Routine typing and virulence determination

In addition to resistance prediction, completely sequenced genomes can provide for epidemiological typing and detection of virulence genes. Analysis of sequence typing and virulence factors in clinically relevant isolates has already been found useful in outbreak investigations.[14,15] Several studies using WGS have revealed that *E. coli* ST131 possess a variable complement of genes encoding established virulence factors in urinary tract infections.[16,17]

For useful application in clinical microbiology, it is necessary to conduct the relevant WGS analysis in realtime. Routine typing, surveillance and outbreak detection of verotoxigenic *E. coli* is already applicable and may also be used for typing and surveillance of other pathogens.[18] The expanded use of WGS in clinical microbiology has the potential to produce the equivalent of a HapMap for microbes (http://hapmap.ncbi.nlm.nih.gov/). When combined with data generated by the human HapMap, the risk of infection by specific strains or clones carrying characterized virulence factors in individuals of a particular haplotype could begin

to be assessed. Even more promising would be simultaneous genotyping of host and bacterial isolate, to define critical host factors in response to infection and progression of disease and treatment tailored to the patient's host characteristics.

7.3.4 Data management and reporting

Although WGS provides detailed information that will, in theory, enable routine diagnostic microbiology solely on the basis of the bacterial genomes, it is a formidable challenge to define and extract the appropriate information from the large amount of sequence data that is generated.[19,20] Thus, to facilitate the use of WGS data for routine diagnostics, typing and surveillance, it is important that the sequence data can be automatically and quickly converted to clinically relevant information that can be easily interpreted without bioinformatics skills. At present, there are several databases for the investigation of antimicrobial resistance genes, including the Antibiotic Resistance Genes Database,[21] Resfinder for acquired antimicrobial resistance genes[22] and Antibiotic Resistance Cassette Database (RAC).[23] For bacterial typing, different comprehensible web-tools for WGS analysis are available, such as the Bacterial Isolate Genome Sequence Database (BIGSDB),[24-26] MLST predictor,[27] and snp-Tree.[28] VirulenceFinder, for detection of *E. coli* virulence genes has been added recently.[29] While most of these databases are new and well updated, they have diverse sources. Reference data should be stored and maintained in one centralized, expandable and up-to-date database, e.g. to include novel resistance determinants.

Predictions about the resistance and susceptibility need to be accurate for proper patient management. One solution is the creation of an international, online database, divided into two parts. The first part is a depository into which accredited diagnostic laboratories routinely deposit WGS data, combined with clinical and epidemiological data, from across the world. These data could provide for an expert panel to discuss and propose new markers for antimicrobial drug resistance or to link genotype directly to disease outcome. Harmonization of these genotypic markers could be modelled in an international up-to-date diagnostic WGS database for routine clinical microbiology laboratories with approved markers for automated WGS data interpretation.

7.3.5 Screening for gastrointestinal colonization

Early detection of multidrug resistant *Enterobacteriaceae* (MDR-E) carriage could allow timely implementation of infection control measures and the appropriate selection of antimicrobials. On-admission surveillance for ESBL-E has been associated with a reduced incidence of MDR-E infections during hospitalization.[30] In 2013, a guideline for the detection of highly resistant microorganisms (HRMO) has been implemented nationally.[31] Patients are placed in contact isolation and screened for HRMO-carriage, if patients are transferred from a foreign hospital, or if they come from any ward with an ongoing outbreak.[31]

Travel to geographic areas with high prevalence of antimicrobial resistant bacteria has been shown to be a risk factor for the acquisition of MDR-E.[32] Prospective cohort studies among travelers returning from Australia, Canada, Sweden and New York revealed high carriage rates of varying from 18-25% after foreign travel [33-36] and showed that travel to India or the Indian subcontinent was the highest risk factor. The study presented in chapter 5 also highlighted international travel as a significant risk factor for the acquisition of MDR-E. The high pre- and post-travel carriage rate of 8.6% and 30.5% of MDR-E respectively as found in chapter 5 has recently been confirmed in another group of 122 returning healthy travelers from the Netherlands.[37] The high rates of ESBL fecal colonization as found in chapter 5 highlights the importance of a targeted screening strategy for Dutch hospitals. The present results implicate that it is necessary to perform active surveillance of MDR-E in patients on admission, who returned from Asia during the previous 6 months, which are not considered to be high risk carriers yet.

Colonization of healthy subjects with antimicrobial resistant *Enterobacteriaceae* could contribute to the amplification of resistance both in the community and nosocomial settings.[38-40] Metagenomic analysis of the fecal microbiome of 162 individuals from Denmark, Spain and China were compared to environmental and agricultural metagenomic data sets. The presence of resistance genes was significantly higher in the gut microbiome, reaching an average of 0.266% of the total number of gut genes. Individuals from China showed the highest diversity of resistance genes, whereas individuals from Denmark showed the lowest levels of diversity and abundance.[41] Another study on the gut microbiome also found a higher abundance of resistance determinants in the microbiota of individuals from Spain, Italy and France compared with individuals from Denmark, the United States and

Japan[42] WGS directly on rectal swabs may allow realtime generation of locally and nationally relevant epidemiological data, allowing strains to be tracked, their relative importance evaluated and control efforts to be meaningfully targeted. Furthermore, the higher level of discrimination will permit the detection and monitoring of newly emerging strains. Linked with clinical surveillance data, this could provide as early warning system.

7.4 Culture-free sequencing

WGS technology is developing at incredible speed and has already transformed the research landscape in microbiology. In the next five years, future developments of workflow and pipelines will facilitate research on antimicrobial resistance mechanisms, genes, virulence markers and epidemiological profiling in a diagnostic setting.[43-45] Full bacterial genomes can already be obtained from a single DNA molecule using the PacBio long-read, single cell molecule, real-time sequencing technology.[46-48] This technique has recently been used to investigate the resistome of cow manure, in which novel and diverse antimicrobial resistance determinants conferring resistance to chloramphenicol, kanamycin, tetracycline, or ß-lactam antibiotics were found.[49]

If we assume that WGS will become increasingly affordable, rapid, and simple to use and that technologies and databases will evolve to the extent that WGS will be highly reproducible and reliable, application in clinical microbiology directly on clinical samples seems a matter of time. Current and realistic future applications of WGS in routine clinical microbiological laboratories are illustrated in Figure 1.

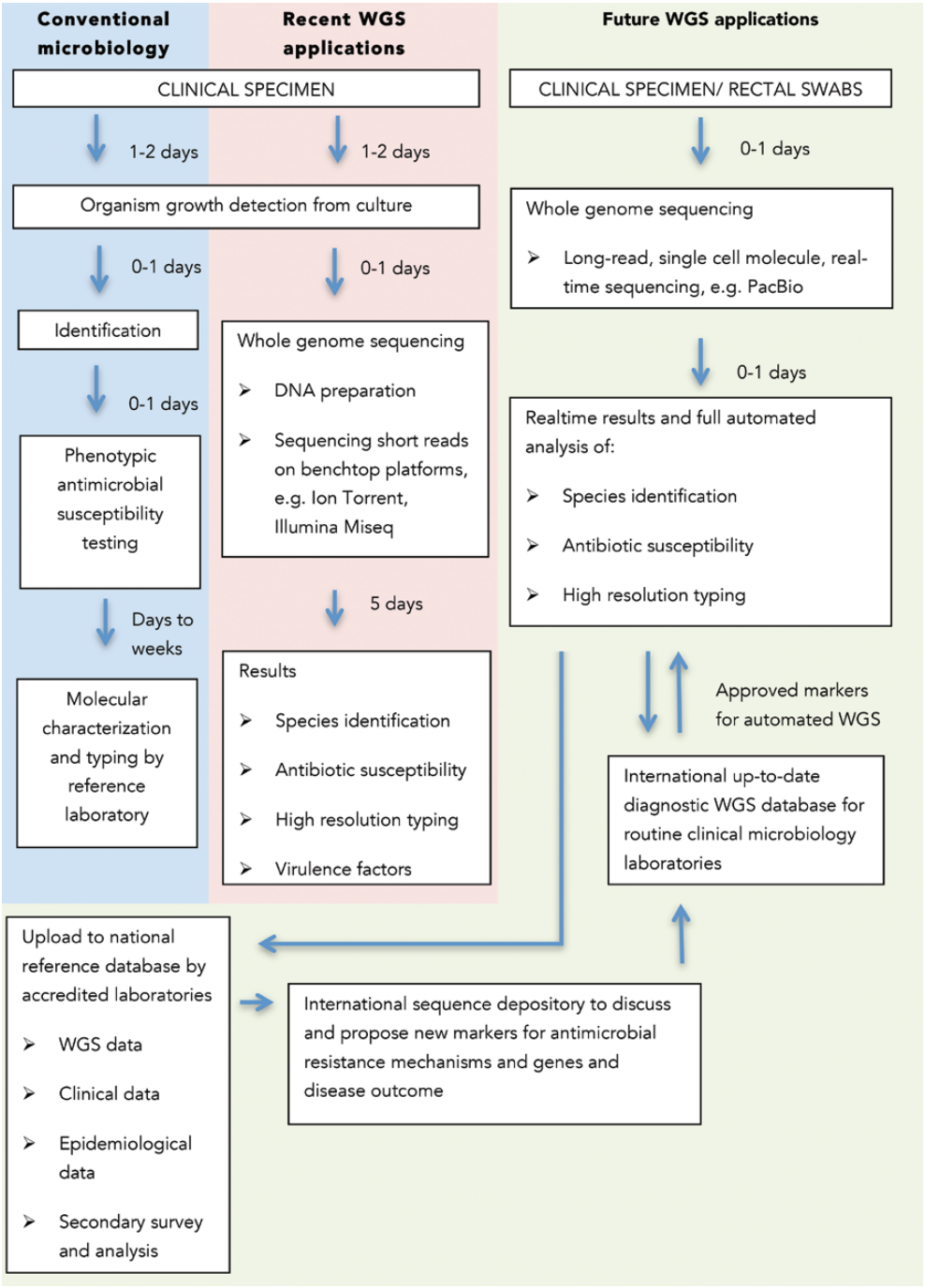

Figure 1. A comparison of the current and future methods in routine clinical microbiology

The eventual adaptation of WGS in every clinical laboratory will provide all information: identification to the species, its susceptibility to antibiotics, its virulence characteristics and epidemiological markers. Because results are obtained rapidly, WGS may ultimately replace most if not all current phenotypical microbiological methods. If WGS would fulfill such expectations, this will revolutionize diagnostics and clinical practice. Empirical antibiotic treatment will be reduced in time to a minimum or tailored therapy may even be started immediately. Furthermore, monitoring and surveillance of antimicrobial resistance could be performed in real-time.

Another promising approach is sequence-based metagenomics, which involves extracting and random sequencing of DNA directly from a variety of environment (e.g. soil, agriculture, livestock, river/sea/lake, aquaculture and the human gut) including DNA from uncultured bacteria.[50-53] The application of metagenomics will not only facilitate future identification of novel resistance mechanisms, but will be used for the prediction of antibiotic resistance and distinct host features, for example in the human microbiome.[54]

To be fully useful, WGS data will have to be shared on local, national and international level; integrated post-genomic approaches to store epidemiological and genomic data are under development.[55, 56] In this respect we could and should learn from micro-organisms who survive thanks to their ability and practice to exchange resistance information rapidly. *Enterobacteriaceae* readily exchange information at a high speed and so should we.

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