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Research Article

Molecular typing of bacteria for epidemiological surveillance and outbreak investigation

Molekulare Typisierung von Bakterien für die epidemiologische Überwachung und Ausbruchsabklärung

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Summary

Constant confrontations with microbial threats pose major challenges to human and animal health, agricultural and food production, and public safety. Identifying pathogenic bacteria (species) and tracking strains (by series of well-characterized isolates) to their sources are especially important in outbreak investigations. Compared to the identification of the species, the identification of the source and spread of microbial infections represents a major—and many times futile—challenge. This is due to the multitude of ways microorganisms can occur and spread within healthcare facilities and in the community; how, when, and where they can contaminate the complex nutrition chain, leading to natural and man-made outbreaks.

Typing is the characterization of isolates or strains below species or subspecies level. Typing of bacterial isolates is an essential procedure to identify the microbe causing the illness or to track down an outbreak to the suspected source. In the genomic era, the introduction of molecular methods has largely replaced phenotypic methods and "molecular epidemiology" has emerged as a new discipline. The current molecular typing methods can be classified into three categories: (a) PCR-based methods, (b) DNA fragment analysis-based methods, and (c) DNA sequence-based methods, including the new exciting era of high-throughput genome sequencing.

Keywords: typing, genetic fingerprint, molecular epidemiology, public health

Zusammenfassung

Die ständige Bedrohung durch Mikroorganismen stellte eine große Herausforderung für die Gesundheit von Mensch und Tier, für die landwirtsschaftliche Produktion, die Lebensmittelproduktion und die öffentlichen Sicherheit dar. Die Identifizierung pathogener Bakterienarten und die Rückverfolgbarkeit von Bakterienstämmen (auf Grundlage gut charakterisierter Isolate) zur Infektionsquelle sind für die Aufklärung von Krankheitsausbrüchen und von Übertragungswegen unbedingt erforderlich.

Im Vergleich zur Artbestimmung stellt die Identifizierung der Infektionsquelle und die Verfolgbarkeit der Ausbreitung von Infektionskrankheiten eine viel größere und oft vergebliche Herausforderung dar. Ursache dafür ist die Unberechenbarkeit von Mikroorgansismen: wie, wann und wo sie sich in Gesundheitseinrichtungen und in der Öffentlichkeit ausbreiten, Nahrungsketten kontaminieren und schließlich natürliche oder durch Menschen verschuldete Ausbrüche verursachen. Unter Typisierung versteht man die Charakterisierung von Bakterienisolaten, oder Bakterienstämmen innerhalb einer Art oder Unterart. In den letzten Jahren haben molekularbiologische Methoden phänotypische Methoden weitgehend verdrängt und mit der "molekularen Epidemiologie" ist eine neue Disziplin entstanden. Molekulare Techniken haben die Artbestimmung und die Charakterisierung von Mikroorganismen deutlich vereinfacht. Molekulare Typisierungsmethoden können in drei Kategorien unterteilt werden: (a) PCR-basierte Methoden, (b) DNA Fragmentanalyse basierte Methoden und (c) DNA Sequenz basierte Methoden einschließlich der Gesamtgenomsequenzierung.

Schlagworte: molekulare Epidemiologie, Typisierung, genetischer Fingerabdruck, Ausbruchsaufklärung, öffentliche Gesundheit

1. Introduction

In the middle of the 20th century, enthusiasm about the success in the fight against infectious diseases caused by the introduction of antibiotics, improved hygienic measures, better nutrition, the availability of clean drinking water, and other factors led to the assumption that bacterial infections were no longer a serious health threat. First cracks appeared almost unperceived in the protective shield against infectious diseases. Currently, bacterial infections represent one of the most important public health issues, including emerging and reemerging bacterial diseases, food-borne and water-borne infections, hospital-acquired infections, and the problem of antibiotic resistance (Galloway et al., 2015). According to the World Health Organization, infectious diseases account for 18% of deaths per year globally (WHO, 2013; Dye, 2014).

Serious infectious disease agents in the pre-antibiotic era such as *Streptococcus pyogenes, Corynebacterium diphtheriae, Staphylococcus aureus, Streptococcus pneumoniae,* and *Enterococci* were uncommon pathogens for decades because of the antibiotic treatment, but reemerged fiercely (Howe et al., 1996; Arias and Murray, 2013). The use and abuse of antimicrobial agents fostered the evolution and selection of bacteria toward intrinsic or acquired resistance (Courvalin, 1996; Waclaw, 2016). For a long time, the warnings from experts were mostly ignored. Funding for antibiotic research was largely cut because the scientific community thought that everything about antibiotic resistance had been revealed (Wilson et al., 2011). Research on and development of new antibiotics was reduced, mainly because of the high costs. In the 1990s, when *S. aureus* became the most common cause of hospital-acquired infections (Swartz et al., 1994) lurid headlines in diverse media on the appearance of rapidly evolving resistant bacterial clones made the problem public. Today, antibiotic-resistant microorganisms in hospitals as well as in the community, agriculture and animal breeding present an ever-increasing worldwide problem (Tenover, 2001; Galloway et al., 2015). It is a fact that infections caused by resistant or multiresistant strains create an additional burden on healthcare systems, rather than merely replacing infections caused by susceptible bacteria (de Kraker et al., 2012). Thus, the emergence of multiresistant bacterial strains deteriorates future prospects in the fight against infectious diseases.

Another unforeseen incidence was the appearance of the so-called emerging and reemerging infectious diseases, representing a serious challenge for future human as well as animal health, agricultural production, and public safety (Morens et al., 2004; Fauci et al., 2005; Becker et al., 2006; Jones et al., 2008; Cutler et al., 2010; Keesing et al., 2010; Lin et al., 2012; Johnson et al., 2015). More than 60% of emerging and reemerging human pathogens are zoonoses, that is, pathogens transmissible between different species (Jones et al., 2008; Cutler et al., 2010; Kilpatrick and Randolph, 2012; Van Doorn, 2014). The major zoonotic diseases from more than 800 are anthrax, animal influenza, bovine spongiform encephalopathy (BSE), food-borne zoonoses (campylobacteriosis, *Escherichia coli*, Salmonellosis, listeriosis, shigellosis, and trichinellosis), hemorrhagic fevers, leptospirosis, prion diseases, and tularaemia (WHO, 2004). More than 70% of these zoonotic diseases in humans originate from wildlife (Morse et al., 2012; Van Doorn, 2014).

These shifts in infectious diseases are caused by the adaptation of microorganisms to changes in human behavior, demographics, and life style (Cascio et al., 2011); changes in economic development and land use (Suhrcke et al., 2011); loss of biodiversity (Swaddle and Calos, 2008; Ostfeld, 2009); global travel (Hufnagel et al., 2004); immigration (Schmid et al., 2008); air conditioning; crowded intensive care units in large hospitals; global environmental and climate changes (Semenza et al., 2012); evolution of susceptible populations, exotic pets, exotic foods and pathogen adaptation (Casadevall et al., 2011; Price et al., 2012); as well as advances in detection techniques (Chan et al., 2010; Allerberger, 2012; van Doorn, 2014).

With industrialization of food processing, worldwide shipment of fresh and frozen food and an increased demand for fresh bagged produce (Allerberger et al., 2015) food-related outbreaks shifted from local, often family-based, outbreaks to multistate or multicountry outbreaks, often caused by a single source (Shane et al., 2002; Tauxe, 2002; Denny et al., 2007; Nygren et al., 2013; Schmid et al., 2014; Ruppitsch et al., 2015b; Inns et al., 2016).

Disease surveillance is an inevitable cornerstone for early identification of infectious disease outbreaks and for timely implementation of accurate measures to combat transmission and morbidity (Johns et al., 2011). Today, where pathogens easily cross national borders, the monitoring of diseases requires an efficient local, national, and international surveillance system. Since the first epidemiological outbreak investigation in 1854 by John Snow (Lilienfeld and Lilienfeld, 1984), our knowledge of microorganisms and epidemiology has largely improved. However, effective outbreak investigation still presents a challenge. The detec-

tion, notification, and verification of an outbreak can take 3 weeks and even longer, if the public health infrastructure fails or is weak and political pressure or fear of economic implications may prevent proper reporting (Chan et al., 2010). At the current stage of scientific knowledge, it is not possible to predict future outbreaks and pathogenic agents will certainly continue to surprise health authorities (Palm et al., 2012; Price et al., 2012).

A relatively new aspect in epidemiology is the recent expansion of Internet data sources for outbreak alert and response (Morse, 2014), such as ProMED-mail (Madoff et al., 2004), FrontlineSMS (Frontline SMS, 2010), HealthMap (Brownstein et al., 2008; Freifeld et al., 2008; 2010), MedISys (http://medisys.newsbrief.eu), GPHIN (www.phac-aspc.gc.ca/gphin), and AFHSC-GEIS (Johns et al., 2011), which may push governments to more transparency and may complement traditional public health infrastructure. Finally, these Internet data sources could accelerate implementing appropriate measures by health authorities. In response to the increase in emerging and reemerging disease threats, the International Health Regulations (2005) (IHR), a binding instrument of international law, became effective in 2007 (WHO, 2008).

The Austrian Agency for Health and Food Safety (AGES) acts as a national focal point for the European Centre for Disease Prevention and Control (ECDC) in Stockholm, Sweden. Data submitted to ECDC are considered to be the proprietary of the individual member states and cannot be shared with the above-mentioned open access media. It is up to the Health and Consumer Protection Directorate General (DG Sanco) of the European Commission and to the individual member state to share information with public media. *Eurosurveillance*, the official journal of ECDC; *Wiener Klinische Wochenschrift*; and other journals are generally used to distribute epidemiological information of interest to the scientific community (Huhulescu et al., 2007; Ruppitsch et al., 2007c; Schmid et al., 2007; 2008; 2009; Pichler et al., 2009; Fretz et al., 2010a; 2010b).

The basis for efficient surveillance is rapid identification of dangerous microbial strains and data sharing (Morse, 2014; Varan et al., 2015). Recent advances in diagnostics facilitate identification and characterization of microorganisms by more accurate and effective tools such as polymerase chain reaction (PCR), real-time PCR, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), Sanger sequencing, and whole genome sequence (WGS) analysis (Figure 1) (Baker et al., 2010).

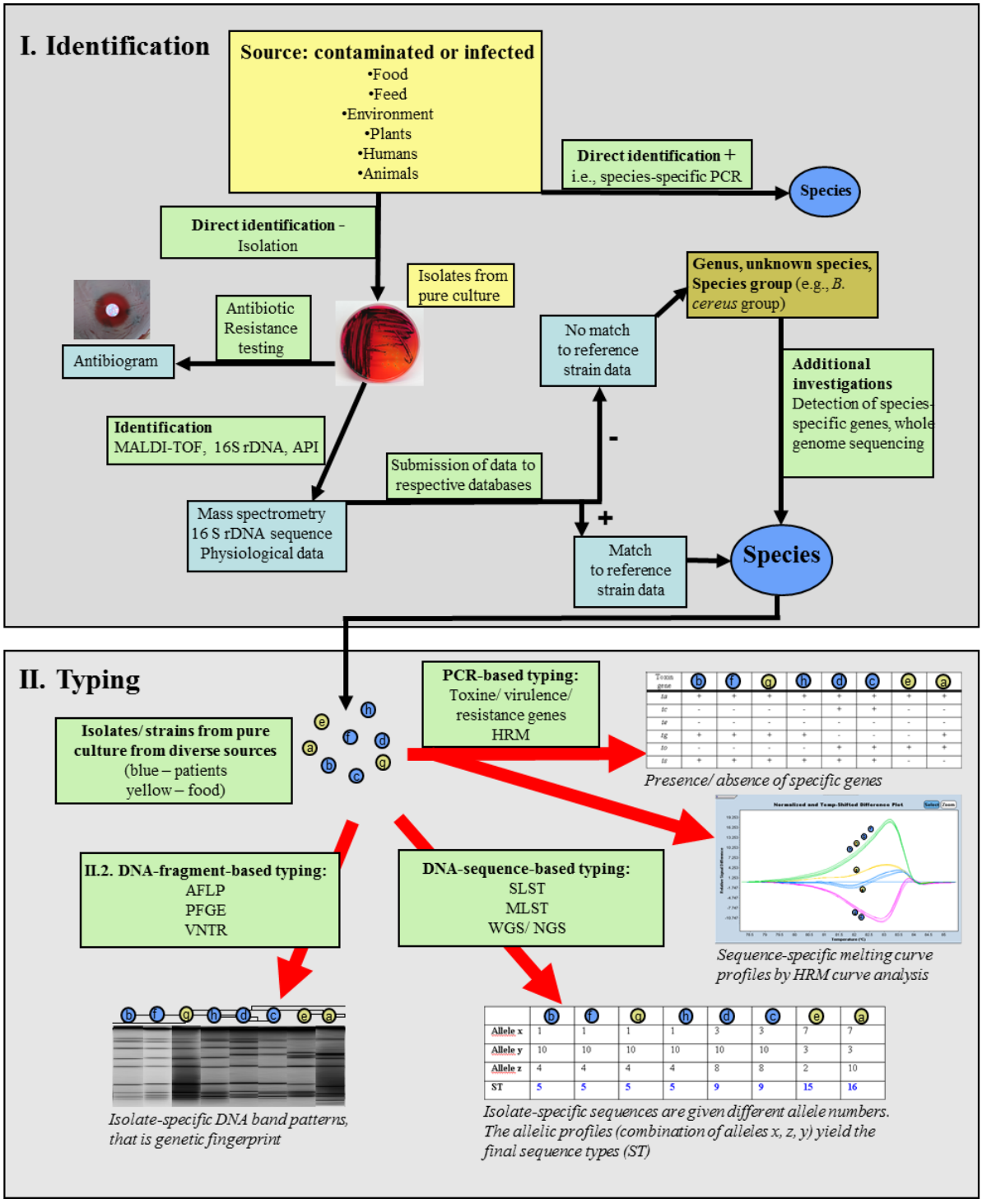
Typing discriminates isolates or strains below species or subspecies level. This is achieved through the application of a multitude of methods comprising DNA-fragment-based, DNA-sequence-based, and PCR-based typing methods. The potential of MALDI-TOF MS for typing has still to be confirmed (Giebel et al., 2010; Kok et al., 2013). Typing results can identify the source of an outbreak, allow study of the microbial population dynamics, and are helpful in epidemiological surveillance of bacterial infections. Besides the ability of a typing method to clearly identify isolates that are involved in an outbreak, the method must accurately differentiate outbreak strains from non-outbreak isolates. However, despite progress and exciting innovations in molecular microbiology, the process of typing in diagnostic laboratories is still laborious and time consuming (Palm et al., 2012). For example, although WGS allowed determination of the genome sequence of the Shiga toxin-producing *E. coli* O104:H4 within days, traditional microbiological methods were indispensable for identifying and characterizing this outbreak strain (Scheutz et al., 2011).

Nevertheless, technological advances in whole genome research will not only raise analysis of pathogen genomes to a new dimension but will also allow analysis of human genomic variations that affect immunity (Casanova and Abel, 2007; Rowell et al., 2012). Currently, research interests in infectious diseases are still underrepresented in human genome epidemiology (i.e., susceptibility to infections reliant on ethnic group and gender), although infectious diseases are a leading cause of morbidity worldwide (Rowell et al., 2012; Palm et al., 2014).

The aim of this outline is to review the value of strain typing in public health and in the food industry. Selected examples are used to give an overview on current molecular typing tools, discussing the potential as well as the shortcomings of diverse techniques in reference to our own work and to present an outlook on upcoming technologies based on WGS.

2. Identification of microorganisms

Identification denotes the assignment of a microorganism into a classification scheme based on diverse criteria to species or subspecies level (Figure 1). In principle, this is a well-established process in microbiological laboratories. In clinical medicine, rapid and accurate pathogen identification is the basis for correct diagnosis and proper treatment.



	b	f	g	h	d	c	e	a
Allele x	1	1	1	1	3	3	7	7
Allele y	10	10	10	10	10	10	3	3
Allele z	4	4	4	4	8	8	2	10
ST	5	5	5	5	9	9	15	16

Figure 1. Outbreak investigation: The first step in outbreak investigation is the isolation and identification of the respective pathogen from a suspected source as well as from patients. Next, the identified isolates are characterized below species level by diverse typing methods. Typing results linked with epidemiological data may lead to the identification of the source of infection. In this example, the food isolate g sharing the same genetic fingerprint, sequence type, melting curve profile, and toxin gene profile with isolates b, f, and h from humans might be the source of infection.

Abbildung 1. Ausbruchabklärung: Der 1. Schritt ist die Isolierung und Identifizierung des Pathogens aus der verdächtigen Probe und von Patienten. Danach werden die identifizierten Isolate durch verschiedene Methoden typisiert. Eine Kombination von Typisierungsergebnissen mit epidemiologischen Daten kann schließlich zur Identifizierung der Infektionsquelle führen. Im Beispiel hat das Lebensmittelisolat g den gleichen genetischen Fingerabdruck wie die Humanisolate b, f und h und ist damit die mögliche Infektionsquelle.

In the microbiology laboratory, technological evolution is changing the identification process from conventional phenotypic techniques to PCR- and sequence-based methods. However, in contrast to viral diagnostics, culture-based methods are still dominating in clinical microbiology (Mancini et al., 2010). Over the past years, sequence analysis of the 16S rRNA gene has allowed identification of several new bacterial species or bacterial communities (Woo et al., 2008; Loncaric et al., 2011) and represents the "gold standard" for bacteria classification because of quality and accuracy of a sequence-based method and the availability of comprehensive databases such as the Ribosomal Database Project (RDP) (Cole et al., 2014) containing 3,356,809 16S rRNAs by November 2016 (rdp.cme.msu.edu) or Greengenes (DeSantis et al., 2006) (http://greengenes.secondgenome.com), which currently allow the identification of more than 15,000 bacterial species (http://www.ncbi.nlm.nih.gov/Taxonomy) (Euzéby, 1997; Parte, 2014). In general, public databases such as Basic Local Alignment Search Tool (BLAST) or the RDP yield better identification results for rare or highly pathogenic bacteria than quality-controlled databases such as RIDOM or MicroSeq 500 (Ruppitsch et al., 2007b). A drawback of 16S-rDNA-based identification is the inability to differentiate some closely related species within the genera *Brucella* (Gee et al., 2004), *Bacillus, Yersinia* (Ruppitsch et al., 2007b; Almeida and Araujo 2013), *Shigella* (Thiem et al., 2004), *Listeria* (Ojima-Kato et al., 2016), and the *Mycobacteria tuberculosis* complex (Jung et al., 2016). Members of these pathogen groups must be identified by applying additional species-specific assays (Winchell et al., 2010; Shallom et al., 2011; Antolinos et al., 2012; Stöckel et al., 2012).

For selected pathogens, specific PCR detection assays, including real-time PCR methods, have been developed: they are very powerful, simple, and effective tools for fast detection and identification, even directly from clinical or environmental specimens (Maheux et al., 2013). To bypass the main disadvantage of PCR-based identification technologies, that is, the one primer pair for each pathogen principle, diverse broad range and multiplex PCR protocols have been published to allow detection of the 35 main important pathogens in a single and closed-tube reaction format, considerably shortening the time to result and improving the outcome of patients (Dark et al., 2009; Mancini et al., 2010; Lucignano et al., 2011).

In our laboratory, a high-resolution melting (HRM) PCR assay was developed for rapid and accurate differentiation of highly pathogenic *Yersinia pestis* strains from *Yersinia pseudotuberculosis* and highly pathogenic *Bacillus anthracis* strains from *Bacillus cereus* strains that allowed specific, rapid, and simple identification of these highly pathogenic bacterial species (Ruppitsch et al., 2008). Owing to the specificity of this new PCR technology, this HRM assay even allowed differentiation of the intentionally released *B. anthracis* Ames strain from other *B. anthracis* strains (Ruppitsch et al., 2008). Furthermore, in our laboratory, PCR assays were developed to detect *Erwinia amylovora*, the causative agent of fire blight, a severe plant disease affecting the sub-family *Pomoideae* and *Xanthomonas fragariae*, which causes angular leaf spot on strawberry plants (Stöger and Ruppitsch, 2004; Stöger et al., 2006). These assays provide higher sensitivity, higher throughput, and easier sample preparation than other methods and are ideal tools for screening of large numbers of samples for both producers and federal control organizations.

3. Typing of microorganisms

Typing is the differentiation of isolates or strains (well-characterized isolates) below species or subspecies level (Figure 1). In past years, molecular biological methods have replaced conventional methods in infectious disease epidemiology, yielding the discipline of "molecular epidemiology" (Foxman and Riley, 2001). Molecular bacterial typing generates isolate-specific genetic fingerprints suitable for assessing epidemiological relatedness.

The molecular basis for typing is the genomic difference between genomes, particularly single nucleotide polymorphisms (SNPs), and insertions and deletions. All genetic variations can be detected by several methods, that is, PCR based, DNA fragment based, sequence based or WGS based. However, the typing resolution differs between the different methods.

3.1 PCR-based typing methods

For rapid typing of isolates in routine diagnostics, PCR-based methods targeting a single genetic region are currently the most powerful techniques in terms of cost, simplicity, and turnaround time with potential for standardization (Figure 2). All other molecular-based techniques, either DNA fragment based such as pulsed-field gel electrophoresis (PFGE) (Schwartz et al., 1983), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), restric-

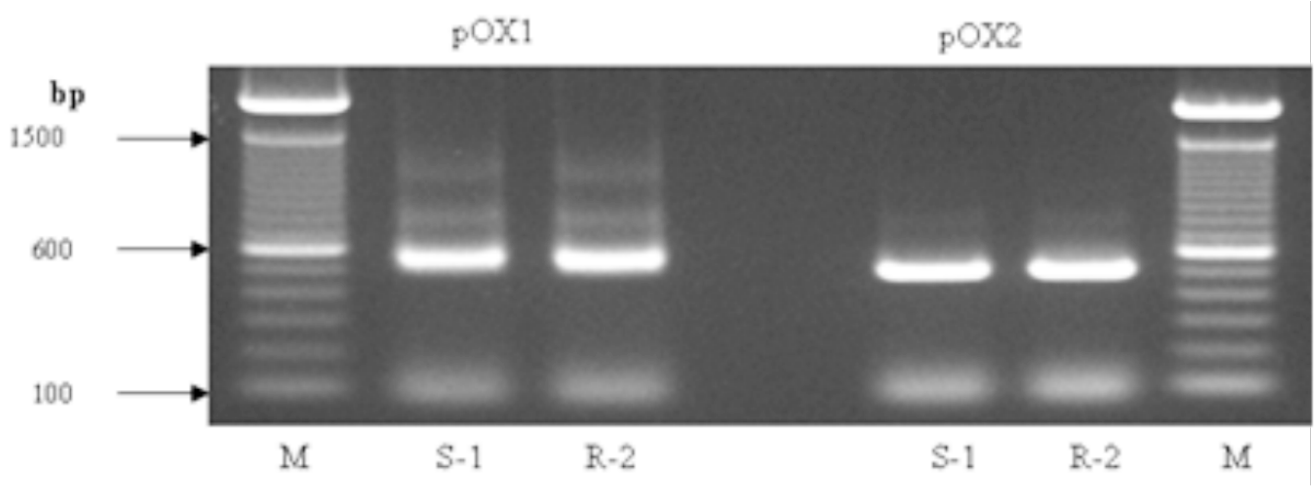


Figure 2. Detection of virulence plasmids pOX1 and pOX2 using plasmid-specific primer pairs, which are species specific for *Bacillus anthracis*. S, isolate from sample; R, reference strain; M, 100 base pair molecular weight marker.
Abbildung 2. Nachweis der *Bacillus anthracis* spezifischen Virulenzplasmide pOX1 und pOX2 mit Plasmid spezifischen Primern. S, Isolat aus Untersuchungsmaterial, R, Referenzstamm, M, 100 Basenpaar Molekulargewichtsmarker.

tion fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD) or sequence based such as multi-locus sequence typing (MLST) (Maiden et al., 1998) , multi-virulence-locus sequence typing (MVLST) (Zhang et al., 2004), and WGS (Köser et al., 2012) are more time consuming and more cost-intensive approaches (van Belkum et al., 2007; Köser et al., 2012). A disadvantage of the PCR-based method is the fact that for each target, you need a specific pair of primers. Multiplex PCR assays may circumvent this shortcoming via parallel analyses of several loci.

3.1.1 Toxin, virulence, and antibiotic resistance gene profiles typing

For particular pathogens (*Vibrio cholerae, E. coli, S. aureus*), identification or typing might be insufficient and toxin and virulence gene data are required to assess their hazardous nature (Table 1) (Monaghan et al., 2011; Schmid et al., 2013).

Toxigenic *V. cholerae*, the cause of cholera, a severe dehydrating diarrhea is a diverse species that comprises strains with a varying pathogenic potential. *V. cholerae* is a common resident of brackish warm water habitats worldwide but can also grow in fresh water (Morris, 2011). Out of more than 200 serotypes that have been described for *V. cholerae*, only serotypes O1 and O139 have caused epidemic and pandemic outbreaks (Faruque et al., 1998). Non-toxigenic forms of *V. cholerae* have been isolated in Austria from the Lake Neusiedl (Huhulescu et al., 2007) and other European countries (Roux et al., 2015). Several reports have described the existence of environmental isolates closely related to O1 and O139 types. A set of virulence genes is required for pathogenicity of *V. cholerae*. Virulence genes can also be found in nonvirulent environmental strains. New virulent strains can emerge with the genetic transfer of virulence genes to these avirulent strains (Faruque and Mekalanos, 2003). Thus, the screening of environmental strains of *V. cholerae* for virulence and toxin genes is of importance especially in nonepidemic areas (Kirschner et al., 2008). Our laboratory serves the National Vibrio Reference Laboratory by providing this virulence typing data (Huhulescu et al., 2007; Bhowmick et al., 2009). Our results demonstrated that all environmental *V. cholerae* isolates from the lake Neusiedler See were non-O1/non-O139 and did not harbor the CTX gene cluster and the vibrio pathogenic island (VPI) that is essential for toxigenicity of epidemic and pandemic *V. cholerae* strains (Huhulescu et al., 2007, Kirschner et al., 2008).

In addition, our laboratory performs toxin gene profiling in cases of *S. aureus*-related food poisoning (Schmid et al., 2007; 2009). We demonstrated that toxin gene profiles yield relevant information, in addition to other typing data in staphylococcal food-borne outbreak investigation (Schmid et al., 2009). Toxin gene profiles revealed that

S. aureus isolates in two acute care hospitals in Austria harboring the new *spa* type t2023 emerged from isolates harboring *spa* type t001, which is the Southern German MRSA prototype, also frequently found in Austria (Ruppitsch et al., 2007a).

3.1.2 High-resolution melting curve (HRM) PCR

The development of high-resolution analysis of DNA melting curves represents a considerable improvement in PCR analysis. This new, simple, rapid, and precise PCR technique allows detection of mutations throughout the entire amplification product (Wittwer et al., 2003; Reed et al., 2007; Ruppitsch et al., 2008; Pietzka et al., 2009; 2010; 2011; Antolinos et al., 2012; Mayerhofer et al., 2012; Zeinzinger et al., 2012), yielding considerably more information than conventional PCR or probe-dependent classical real-time PCR genotyping methods. The specificity of single nucleotide polymorphism (SNP) detection using HRM curve technology is comparable to DNA sequencing. The potential of HRM curve technology to detect diverse mutations within a DNA fragment in a single and simple PCR step makes the method very powerful for SNP-based high-throughput typing applications (Figure 3).

The usefulness of this technique was demonstrated during a multinational outbreak of listeriosis in 2009 and 2010 affecting Austria, Germany, and the Czech Republic, where a newly developed internalin B gene scanning assay was used successfully for screening more than 100 food isolates for source identification (Fretz et al., 2010a; 2010b). In this outbreak, HRM curve profiling allowed unambiguous differentiation between two different clones, as well as immediate identification of new cases as either outbreak related or unrelated (Fretz et al., 2010a).

Table 1. Detection of diverse toxin and virulence genes by PCR. VPI, vibrio pathogenic island; (+) target is present; (–) target is absent. The absence and presence of targets yield isolate-specific binary codes.
Tabelle 1. Nachweis von Toxin- und Virulenzgenen mittels PCR. VPI, Vibrionen pathogenitätsinsel, (+) Target ist vorhanden, (-) Target fehlt. Die Anwesenheit und das Fehlen vonTargets ergibt Isolat spezifische Binärcodes.

Strain ID	VPI						Serogroup		CTX			toxR	Toxine genes			
	tcpI	tcpA (class.)	tcpA*	vpi R	ssrA	vpi L	Vc-O139	Vc-O1	ctxA	zot	ace		st	hlyA (class.)	hlyA *	ompU
CHT 18/01	+	+	-	+	+	+	-	+	-	+	+	+	-	-	-	+
CHT 57/03	+	+	-	+	+	+	-	+	-	+	+	+	-	-	-	+
CHT 17/01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHT 59/04	+	-	+	+	+	+	-	+	-	+	+	+	-	+	+	+
CHT 60/04	+	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+
W 15/EU 158	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	-
W 16/EU 156	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	+
W 27/CHT 71	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
CHT 14/00	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
CHT 15/00	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
CHT 16/01	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
CHT 19/01	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
CHT 20/01	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CHT 54/02	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	+
CHT 55/02	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
CHT 61/04	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	+
CHT 62/04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHT 63/05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHT 64/05	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	-
CHT 67/05	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	-
CHT 68/05	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

HRM was used further for rapid identification of multidrug-resistant (MDR) *M. tuberculosis* isolates by mutation scanning of the cluster I site within the *rpoB* (Hoek et al., 2008; Pietzka et al., 2009; 2010); for rapid subtyping of the hypervariable region X of the protein A gene (*spa*) of methicillin-resistant *S. aureus*, one of the most significant healthcare-associated pathogens (Mayerhofer et al., 2015); and for rapid subtyping of 39 *Salmonella enterica* serotypes, which cover more than 94% of all human and more than 85% of all nonhuman (i.e., food-, environmental-, and animal-derived isolates) *S. enterica* isolates in Austria using a triplex gene scanning assay (Zeinzinger et al., 2012).

3.2 DNA-fragment-analysis-based methods

3.2.1 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a typing technique based on the amplification of genomic restriction fragments via PCR (Figure 4) (Vos et al., 1995). AFLP represents a relatively simple, low cost, rapid, and highly discriminatory method that covers a larger portion of the genome than other typing techniques (Vos et al., 1995; Szaluś-Jordanow et al., 2013). No prior knowledge of the DNA sequence of the respective organism is necessary (Pietzka et al., 2008a; Bhowmick et al., 2009). In principle, the method scans the genome for sequence polymorphisms producing DNA fragments, mainly between 50 and 700 basepairs in size. The presence and absence of fragments produces a band pattern or AFLP profile comparable to bar codes used for product identification in commerce. Here it determines a genetic fingerprint. For subsequent data analysis, the resulting AFLP profile is finally converted into a binary presence or absence (+/– or 1/0) code, a process known as "scoring" (Kück et al., 2012). The bin code obtained is specific for a species and represents the basis for determining the relatedness of strains (Figure 4). AFLP has been very useful for taxonomic studies because it clearly classifies bacteria belonging to the same genomic species and is very discriminative in differentiating highly related bacterial strains belonging to the same species (Lin et al., 1996; Keim et al., 1997). AFLP has been successfully applied to a variety of bacteria, for example, *Pseudomonas* (Abdul Wahab et al., 2014), *Mycobacterium* (Hoek et al., 2008; Chen et al., 2011), *Lactococcus* (Kütahya et al., 2011), *Listeria* (Lomonaco et al., 2011), *B. cereus* group (Tourasse et al., 2011),

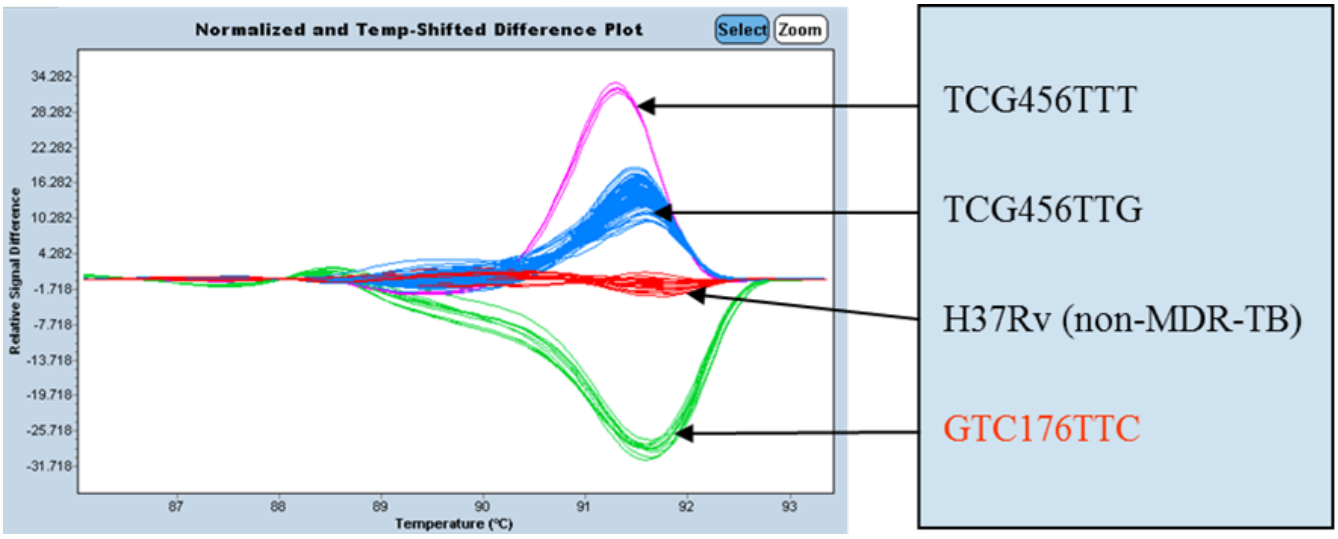


Figure 3. High-resolution melting curve analysis of amplification products of cluster I of the *rpoB* gene of *Mycobacterium tuberculosis* isolates for mutation scanning. The baseline represents the drug-sensitive control. The mutations of the respective drug-resistant isolates are indicated for the different melting curve profiles.
Abbildung 3. Hochauflösende Schmelzkurvenanalyse von PCR Produkten des *rpoB* Gencluster I von *Mycobacterium tuberculosis* Isolaten zur Identifizierung von Mutationen. Die Basislinie zeigt die Antibiotika sensitive Kontrolle. Die Mutationen der entsprechenden Antibiotika resistenten Isolate sind für die verschiedenen Schmelzkurvenprofile angegeben.

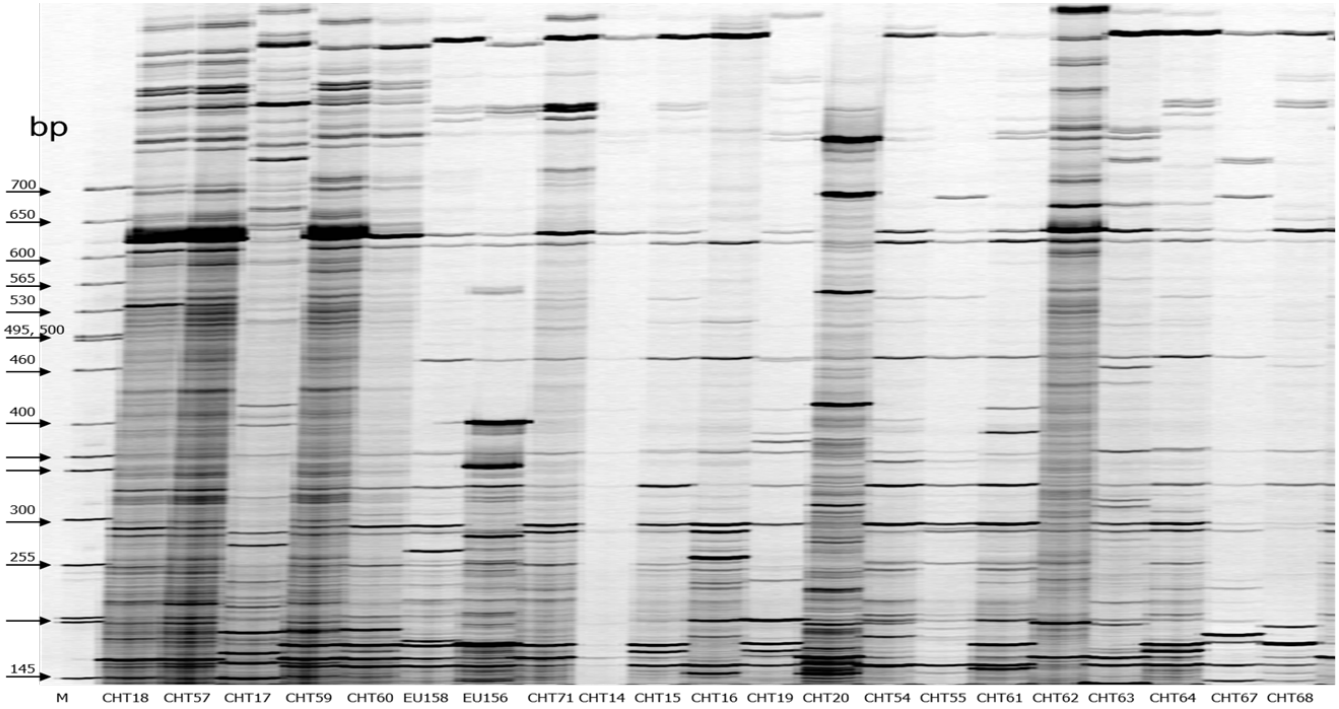


Figure 4. Diversity of *Vibrio cholerae* isolates as determined by amplified fragment length polymorphism. Each isolate is characterized by a specific band pattern. Related isolates display identical band patterns (CHT18, CHT57, CHT59).
Abbildung 4. Diversität von *Vibrio cholarae* Isolaten dargestellt durch Amplifizierte Fragmentlängen Polymorphismen. Jedes Isolat ist durch ein spezifisches Bandenmuster charakterisiert. Verwandte Isolate haben gleiche Bandenmuster (CHT18, CHT57, CHT59).

Salmonella (Romani et al., 2007), *V. cholerae* (Mishra et al., 2011), and *Klebsiella* (Donnarumma et al., 2012).
Our laboratory applied this method for typing of *E. amylovora* (Ruppitsch et al., 2006b), *S. enterica* serovars (Pietzka et al., 2008a), *Listeria monocytogenes* (Pichler et al., 2009), *V. cholerae* (Bhowmick et al., 2009), and *Pseudomonas aeruginosa* (Huhulescu et al., 2011). AFLP could cluster outbreak isolates clearly, differentiating from non-outbreak (Huhulescu et al., 2009) or environmental from clinical isolates (Huhulescu et al., 2007; Pietzka et al., 2008a).

3.2.2 Pulsed-field gel electrophoresis (PFGE)

PFGE was initially described in 1983 (Schwartz et al., 1983) and still represents the "gold standard" in molecular typing of most bacterial species. PFGE is a RFLP-based gel electrophoresis method in which genomic DNA is restricted with rare cutting restriction endonucleases (macrorestriction) yielding a limited and separable number of high molecular weight DNA fragments. The fragments are finally separated in an electric field where the orientation is changed periodically (pulsed-field) (Figure 5). The development of this method overcame the limitations of conventional electrophoresis by separating large DNA fragments. A common problem of gel-based fragment analysis method is that, in principle, only patterns generated on the same gel can be compared directly. To compare data obtained from different runs or— even worse—from different laboratories, strict protocols must be applied (Swaminathan et al., 2001). Owing to its high discriminatory power, PFGE is the method of choice in outbreak investigation, although faster and simpler methods are highly appreciated (Ruppitsch et al., 2007a, 2007c; Fretz et al., 2010a, 2010b; Huhulescu et al., 2011; Chung et al., 2012). In our laboratory, PFGE analysis was applied for epidemiological investigation and elucidation of a multinational listeriosis outbreak in Austria, Germany, and the Czech Republic in the years 2009–2010 (Fretz et al., 2010a, 2010b) and an ongoing outbreak in Germany (Ruppitsch et al., 2015b). Furthermore, PFGE was used to identify the source of infection in a fatal case of *P. aeruginosa* pneumonia in a healthy woman (Huhulescu et al., 2011), and for an in-depth analysis of isolates with—at that time— a new *spa* type t2023 occurring in two acute care hospitals in Austria. Isolates harboring *spa* type t2023

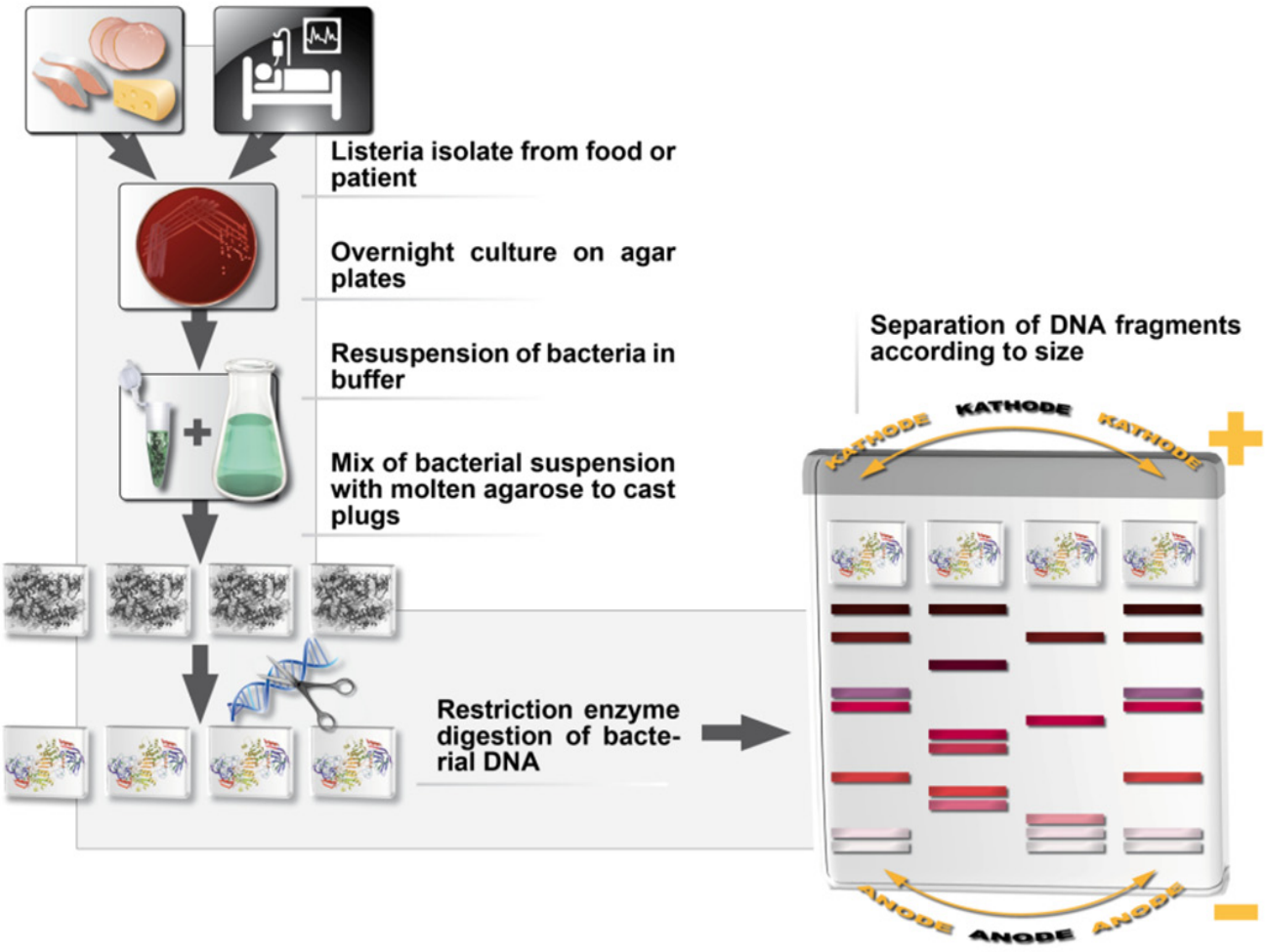


Figure 5. Schematic presentation of PFGE analysis for *Listeria monocytogenes* (from Allerberger et al., 2015; with permission from Springer Verlag, Heidelberg).
Abbildung 5. Schematische Darstellung der PFGE Analyse von *Listeria monocytogenes* (aus Allerberger et al., 2015; mit Genehmigung des Springer Verlags, Heidelberg).

and isolates harboring *spa* type t001, which is the Southern German MRSA prototype and frequently found in Austria, shared the identical PFGE profile (Ruppitsch et al., 2007a).

3.2.3 Variable number of tandem repeat (VNTR) analysis

Variable number of tandem repeats (VNTR) or simple sequence repeats (SSR) are reiterations of at least two nucleotides in the genome (Britten and Kohne 1968). The terminology depends on the number of nucleotides within a repeat unit. Microsatellites (SSR or tandem repeats) contain 2–7 nucleotides, minisatellites harbor 10–100 nucleotides, and repeat units with more than 100 nucleotides are termed macrosatellites. The analysis of several loci is named multi-locus VNTR analysis (MLVA). The variations in repeat unit numbers, sizes, sequences, and the possibility to include several loci into the analysis scheme render MLVA a very powerful and highly discriminatory typing technique. MLVA typing schemes have been developed for nearly all pathogenic bacteria (Figure 6) (van Belkum, 2007). The final result is the number of repeat units that is calculated from the fragment size. In our laboratory, SSR analysis was used for typing of *E. amylovora* the etiologic agent of fire blight (Ruppitsch et al., 2004; Barionovi et al., 2006). SSR typing data revealed that the spreading of fire blight from the western provinces to the east of Austria and onward to

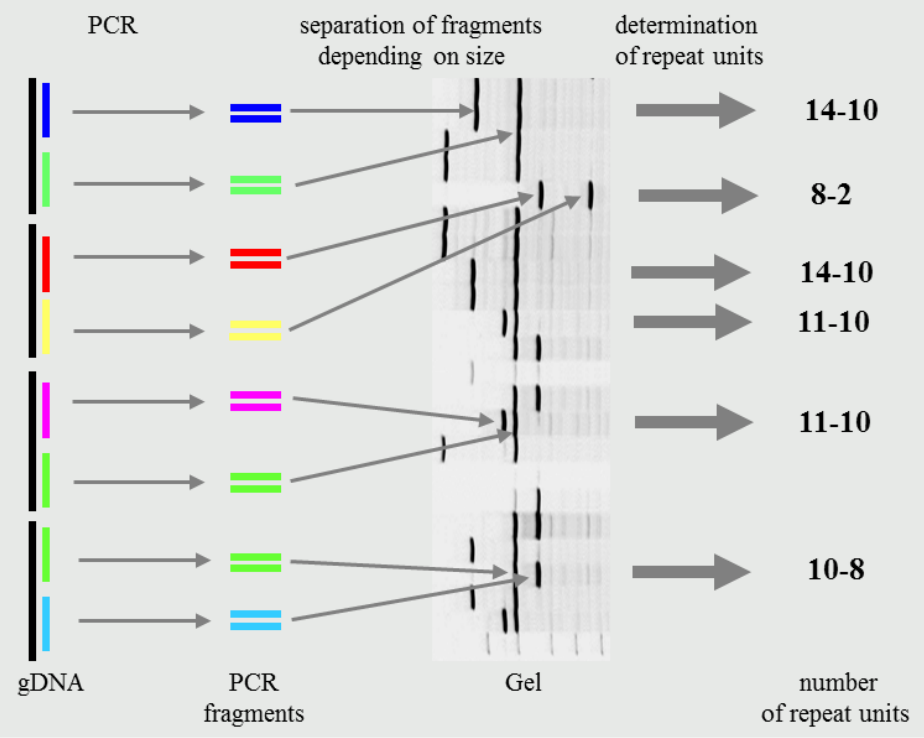


Figure 6. Schematic presentation of MLVA typing. Two different loci for diverse isolates are shown. PCR fragments size (indicated by different colors) depends on the number of repeat units within each repeat locus.
Abbildung 6. Schematische Darstellung der MLVA Typisierung. Zwei Repeatloci für verschiedene Bakterienisolate werden gezeigt. Die DNA Fragmentgröße (angezeigt durch verschiedene Farben) ist abhängig von der Repeatanzahl im jeweiligen Repeatlocus.

Hungary was only an apparently continuous event. Propagation of fire blight in the eastern provinces of Austria was due to several imports of infected plant material or transportation of infected timber for furniture production (Ruppitsch et al., 2004; 2006b). The same is true for Hungary that received fire blight from importing infected plant material (Jock et al., 2002). MLVA typing was used to characterize Austrian *B. anthracis* strains and the isolate from the 2001 bioterrorism attack, which was cultured from mail addressed to the United States embassy in Vienna (Ruppitsch et al., 2008). We were able to demonstrate that the isolate from the mail had the identical MLVA type as the *B. anthracis* Ames strain. Furthermore, the Austrian *B. anthracis* isolates had unique MLVA types but were closely related to *B. anthracis* isolates from mountain regions in northern Italy and France (Ruppitsch et al., 2008).
In the case of tuberculosis surveillance, MLVA has become the gold standard of typing. This MLVA application is generally called mycobacterial interspersed repetitive units (MIRU)—VNTR analysis—and was applied in our laboratory for typing of 1,294 *M. tuberculosis* isolates from Austria (Pietzka et al., 2008b) and outbreak investigation (Schmid et al., 2008).

3.3 DNA-Sequence-based methods

Sequence-based typing methods are the most advanced and accurate techniques currently available for characterization of isolates or strains. Depending on the target and questions that have to be answered in an outbreak or epidemiological investigation, single or multiple genes can or must be analyzed for strain characterization.

3.3.1 Single-locus sequence typing (SLST)

Single-locus sequence typing (SLST) applications is the sequence analysis of a single gene with sufficient sequence variations for typing bacterial isolates (Beall et al., 1996; Frenay et al., 1996; Olvera et al., 2006; Bennett et al., 2007). Typing the polymorphic X region of the staphylo-

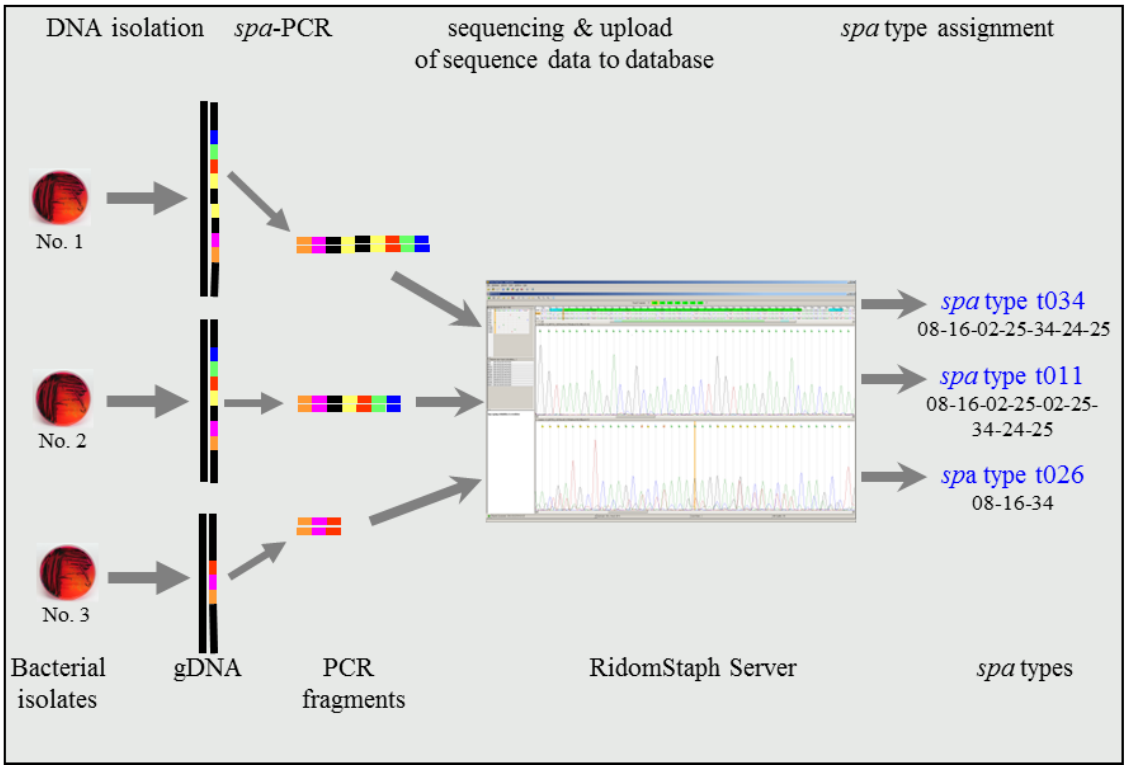


Figure 7. Schematic presentation of *spa* typing of *Staphylococcus aureus* isolates. Repeat units are presented by colors. A spa type is characterized by the order and number of certain repeat units, resulting in a numerical code for repeat units and finally for a given spa type.
Abbildung 7. Schematische Darstellung der *spa* Typisierung von *Staphylococcus aureus* Isolaten. Repeateinheiten sind durch Farben dargestellt. Der spa Typ wird durch die Reihenfolge und Anzahl der Repeateinheiten bestimmt. Für die unterschiedlichen Repeateinheiten und spa Typen werden Nummerncodes vergeben.

coccal protein A gene (*spa*) by DNA sequence analysis is the most advanced example for SLST and also an example of high end SSR analysis (Frenay et al., 1996). Actually, *spa* typing is a variant VNTR analysis, where in addition to the classical VNTR analysis—that is, the determination of the number of repeats of a repeat region—the sequence of this region is included in the final analysis (Figure 7). Owing to the variability in length and sequence of the repeat region of the *spa*, specialized software for automated *spa* type determination, improved quality control of sequence data, and simplified Internet-based data management has been developed (Harmsen et al., 2003). The principle of *spa* typing is the sequence diversity present within the repeat region because of spontaneous single mutations within a repeat unit or deletions or insertions of triplets or entire repeat units (Harmsen et al., 2003). A *spa* type is characterized by the order and number of certain repeat units, resulting in a numerical code for repeat units and finally for a given *spa* type. The repeat region of *spa* is relatively stable, without selective pressure (own unpublished data; Shopsin et al., 1999; Tang et al., 2000). Under selective pressure, up to 10% of strains shows mutations in the variable X-region of the *spa* (van Belkum et al., 1996; Ruppitsch et al., 2006a). This limitation—the variability of the X-region—can be circumvented by classifying *spa* types into *spa* complexes, with respect to SSR unit similarity (Figure 6) (Ruppitsch et al., 2006a; Strommenger et al., 2006). This makes *spa* typing useful for short-term investigations (outbreak investigation) as well as for long-term epidemiological studies (Stöger et al., 2007).

In November 2016, 718 different repeat units and 16,430 *spa* types have been described (http://*spa*server.ridom.de). At the Austrian reference laboratory for staphylococci *spa* typing of 4,500 *S. aureus* isolates from the years 2005 till 2016 yielded 350 *spa* types (personal unpublished data) with different *spa* types dominating in the diverse regions of Austria (Ruppitsch et al., 2006a).

In our laboratory, *spa* typing was successfully used for tracking strain transmission between two hospitals in Austria (Ruppitsch et al., 2007a), to detect the first occurrence

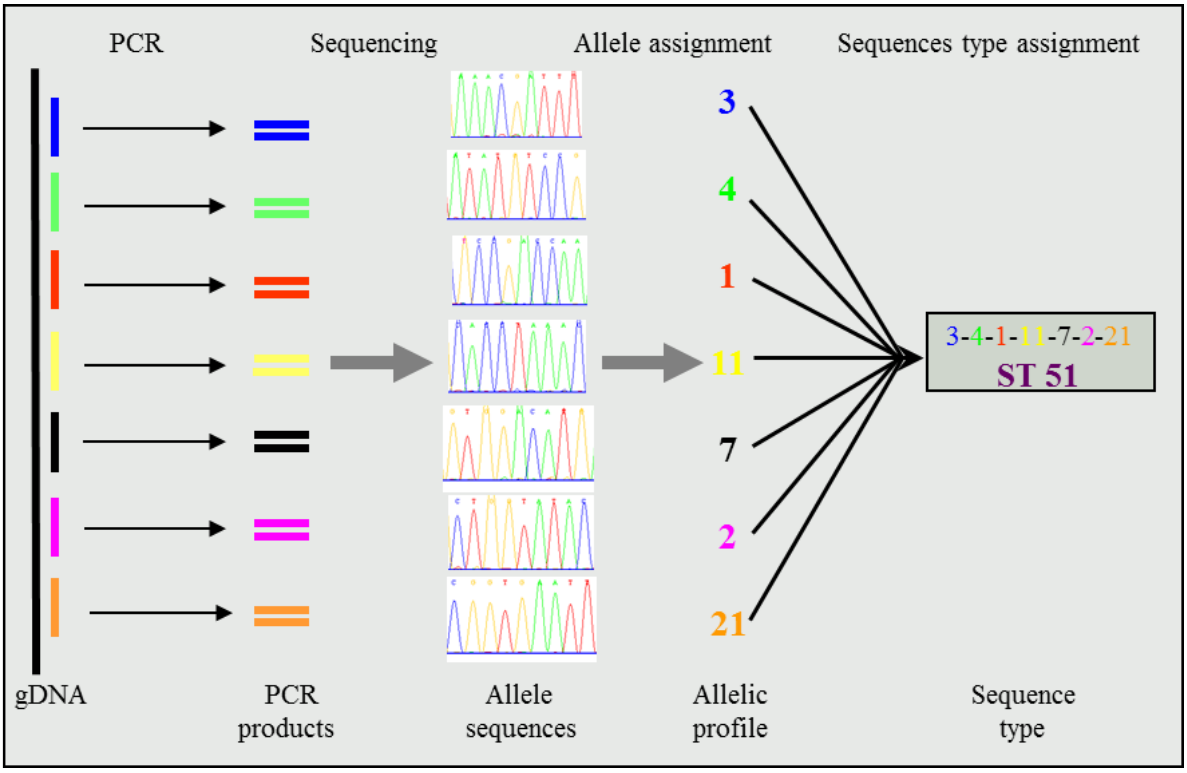


Figure 8. Scheme for multilocus sequence typing adapted from mlst.net. MLST uses sequence variations in up to seven housekeeping genes. Allele numbers are assigned to unique sequences and the allele number combination result in a sequence type.
Abbildung 8. Schematische Darstellung der Multilocus Sequenztypisierung – adaptiert aus mlst.net. MLST beruht auf Sequenzunterschieden in bis zu sieben Haushaltsgenen. Allelnummern werden spezifischen Sequenzen zugeordnet und die Kombination ergibt den Sequenztyp.

of the highly virulent USA 300 strain in Austria (Ruppitsch et al., 2007c), to detect and elucidate food-borne staphylococcal outbreaks in Austrian (Schmid et al., 2007; 2009), to detect the first occurrence of strains of the new zoonotic MRSA clone ST398 in Austria (Springer et al., 2009), and to detect the first nosocomial transmission of a ST398 clone in an Austrian hospital (Schmid et al., 2012).

3.3.2 Multi-locus sequence typing (MLST)

MLST uses sequence variations in up to seven housekeeping genes to characterize bacterial isolates via the Internet (Aanensen and Spratt, 2005) and is generally accepted for the characterization of currently 120 microorganisms (http://pubmlst.org/data). MLST data are accurate and comparable between different laboratories in contrast to gel-based DNA fragment analysis data (Maiden et al., 1998). Aligned and edited sequences of each gene of a strain are submitted to the respective MLST database. Allele numbers are assigned to the unique sequences and the combination of the allele numbers obtained result in a distinct sequence type (ST) (Figure 8) (Maiden et al., 1998). Additional database tools allow worldwide comparison of strains, clustering of STs based on differences in the allele profile, construction of phylogenetic trees based on concatenated sequences, and identification of clonal complexes using eBurst (Feil et al., 2004). Owing to the slow mutation rate of housekeeping genes, MLST often does not display the resolution of PFGE or MLVA, but is the method of choice for long-term epidemiological and phylogenetic investigations. In our laboratory, MLST was used for typing of particular *S. enterica* serovars (Zeinzinger et al., 2012) and we were able to confirm that MLST has the discriminatory power to type isolates beneath serotype level (Achtman et al., 2012; Zeinzinger et al., 2012).

3.3.3 Next generation sequencing (NGS)

The progress in technology from automated Sanger sequencing (first-generation sequencing) to next-generation sequencing (NGS) alias WGS has revolutionized the field of genomics, genetics, as well as microbiology. NGS allows

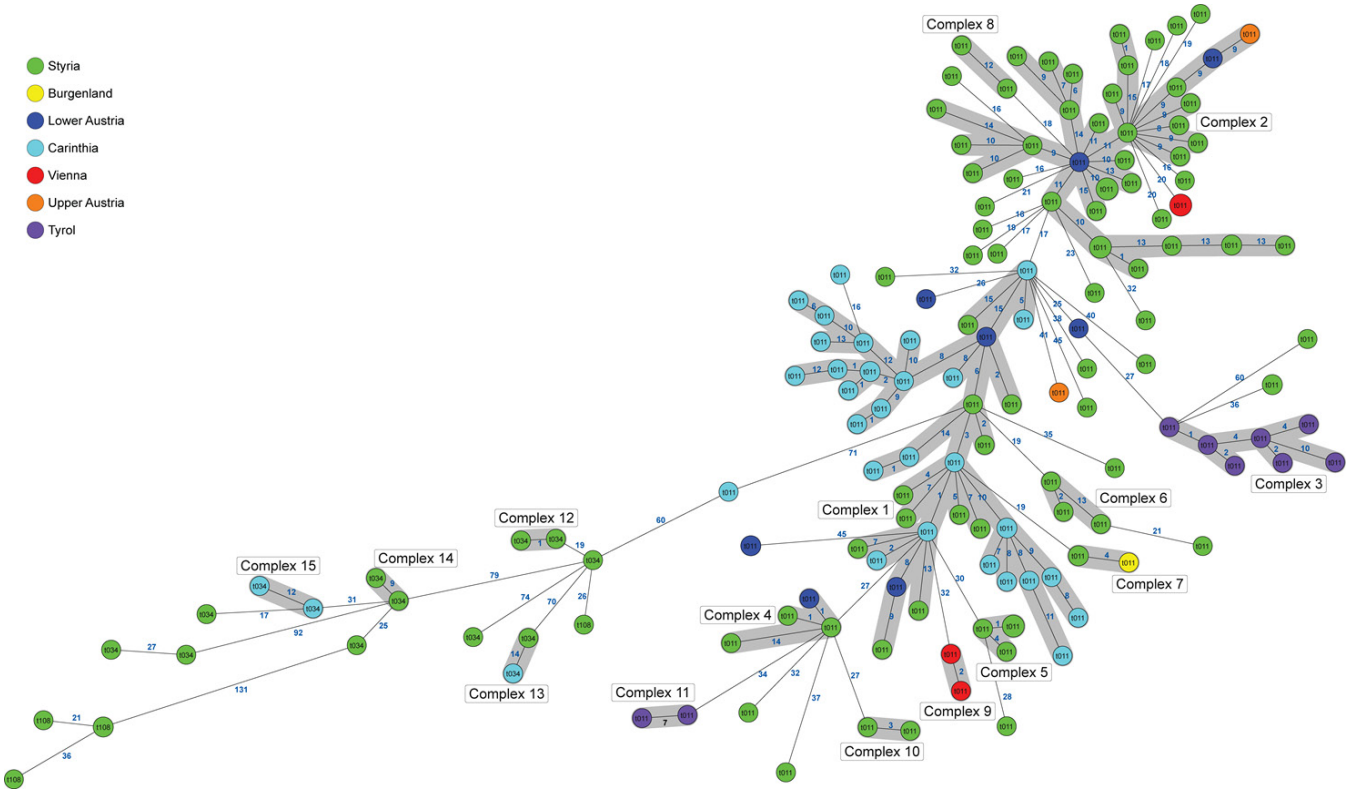


Figure 9. The superior resolution of WGS: Minimum spanning tree for LA-MRSA isolates, all with classical MLST ST398, based on the cgMLST of *S. aureus* consisting of 1,862 alleles. Colors correspond to the province of origin in Austria. Each circle represents isolates with an allelic profile based on the cgMLST. Blue numbers show the allelic differences between two isolates. Clusters of closely related isolates are shaded in gray (Source: Lepuschitz, 2015)

Abbildung 9. Die höhere Auflösung der Gesamtgenomsequenzierung: minimaler Spannbaum für LA-MRSA Isolate, alle mit klassischem MLST ST398, basierend auf dem *S. aureus* Kerngenom bestehen aus 1862 Allelen. Die verschiedenen Farben entsprechen den Bundesländern. Jeder Kreis entspricht Isolaten mit einem bestimmten Genprofil basierend am cgMLST. Blaue Zahlen auf den Linien geben die Anzahl der Unterschiede zwischen Isolaten an. Cluster, bestehend aus ähnlichen Isolaten, sind grau markiert (Quelle: Lepuschitz, 2015).

fast, high-throughput, and, nowadays, with introduction of the third or fourth generation of NGS instruments, also inexpensive analysis of several hundreds of genes or entire (bacterial) genomes within a single day (for approximately 50 €) (Almeida and Araujo, 2012).

The major improvement of NGS/WGS over other sequencing technologies or sequence-based methods is the immense amount of data. The ability to interpret this data represents the current bottleneck of NGS preventing the broad usage in diagnostics (Stone et al., 2012). NGS/WGS platforms exist from Illumina, Thermo Fisher, Oxford Nanopore, and Pacific Biosciences. Although the biochemistry of the diverse platforms might be different, the workflow is similar (Shendure and Hanlee, 2008) including template preparation, sequencing and imaging, genome alignment, and assembly (Metzker, 2010). The advantages as well as disadvantages of diverse platforms (Metzker, 2010; Quail et al., 2012; Doi et al., 2014; Hinrichs et al., 2015; Hahn et al., 2016) have been reviewed.

The most immediate field where NGS have been introduced into daily routine diagnostics in microbiology is surveillance and outbreak investigation. Several studies on a variety of bacterial species have already shown that WGS-based typing, based either on single nucleotide variants (SNVs) (Turabelidze et al., 2013; Pightling et al., 2015) or on gene-by-gene allelic profiling of core genome genes, frequently named core genome MLST (cgMLST) or MLST$^+$ (Laing et al., 2010; Mellmann et al., 2011; Köser et al., 2012; Maiden et al., 2013; Antwerpen et al., 2015; de Been et al., 2015; Moran-Gilad et al., 2015; Chaudhari et al., 2016; Moura et al., 2016), currently represents the ultimate diagnostic typing tool that have been successfully applied for outbreak investigations (Figure 9) (den Bakker et al., 2014; Schmid et al., 2014; Ruppitsch et al., 2015b;

Burckhardt et al., 2016; Chen et al., 2016; Jackson et al., 2016). Backward compatibility of NGS to former methods can be maintained by the extraction of the respective information from NGS data (Hyden et al., 2016). The undeniable advantage of NGS is the optimal resolution that can be achieved by a method for strain typing. With NGS, even small genetic variations occurring in an outbreak strain over the course of an outbreak can be monitored. By tracking the presence or absence of these mutations in all pathogenic genomes from a given outbreak, it is possible to identify where particular variants arose and trace person-to-person transmission events (Köser et al., 2012).

With the development of BIGSdb (Jolley and Maiden, 2010), GenomeTrakR (Allard et al., 2016), and cgMLST.org (http://www.cgmlst.org; Ridom GmbH), global databases that allow the establishment of Internet-based standardized nomenclatures like the current MLST databases have become available (Maiden et al., 1998). The benefit of global databases in infectious disease research is the global exchange of data improving outbreak investigation, strain tracking, and source identification (Ruppitsch et al., 2015b).

4. Conclusion

Although conventional typing techniques such as phage typing or serotyping have largely been replaced by molecular methods because of cost, speed, accuracy, data interpretation, sensitivity, specificity, and simplicity, there is still no general typing method that meets all desirable criteria available. Thus, the method chosen for typing largely depends on the microbiological question (van Belkum et al., 2001). Molecular epidemiology is based on the principle of comparing genotypic characteristics of pathogens. Molecular epidemiology combines data obtained from molecular analysis of strains and isolates with epidemiological data obtained from the detection, investigation, and analysis of infectious diseases in human, animal, or plant populations. As a consequence, the pure molecular analysis of isolates or strains without a linkage to epidemiological data may not be regarded as "molecular epidemiology."

Attribution of a particular pathogenic subtype to an outbreak or to contamination of foods and feeds is often possible, simply by relying on phylogenetic markers. However, indistinguishable typing patterns must not be misunderstood as absolute proof of causative relation, because alternate hypotheses will exist without exception (Keim et al., 2011). Molecular techniques usually provide highest sensitivity and specificity and, therefore, deliver more precise data than phenotypic methods. In the field of epidemiology application of molecular techniques enables detection of new pathogens, disease surveillance, surveillance of outbreaks, identification of transmission patterns, study of host–pathogen interactions, selective effect of genetic polymorphisms in humans by infectious pathogens, association of cancer or other infectious diseases to infective agents, and detection of unculturable microorganisms (Foxman and Riley, 2001).

The key part for accurate diagnosis, for effective and timely treatment of a disease, and subsequently, for the discovery of the source of infection or outbreak is the proper identification of the infective agent (Vlek et al., 2012). The process of identification is well established for known pathogens in microbiological laboratories, and therefore, nearly all clinically important microorganisms can be identified without difficulty. The disadvantage of this system is, however, that we recognize only what we already know. The elucidation of the cause of new and emerging infectious diseases is more challenging and often even impossible when using only conventional methods. The shift from phenotypic to molecular methods has largely improved the identification process and facilitated the detection of yet unknown microorganisms (Relman et al., 1992; Roads et al., 2012).

The final product of this ongoing evolution in diagnostics is the implementation of mass spectrometry for fast and accurate identification of microorganisms. The value of MALDI-TOF MS for typing has to be elucidated (Giebel et al., 2010; Kok et al., 2013). To classify newly discovered microorganisms,16S rDNA sequencing—or more probably in the very near future NGS—will still remain an essential identification tool.

The introduction of these modern technologies in the microbiological laboratories is still slow, because of the traditionally well-established conventional identification methods. However, many traditional methods require days to properly identify certain pathogens, whereas modern molecular methods, in addition to being much faster, display also an improved sensitivity and specificity (te Witt et al., 2010). For the fast and specific detection of pathogens directly from sample material, a specific PCR assay is the state-of-the-art method. The advantage of PCR technology is the specific and fast detection of pathogens directly from specimen without the need for cultivation. The drawback of PCR technology—one specific primer for

each pathogen—was at least partially eliminated through the development of multiplex PCR assay that allows detection of up to 35 different pathogens in a single reaction (Mancini et al., 2010).

The ability to track bacterial outbreak strains accurately is essential for control and prevention of disease. The Antibiotic Resistance, Prevention and Control (ARPAC) project of the European Commission highly recommended the use of molecular typing techniques by national health authorities for alert organisms (MacKenzie et al., 2005). In cases of outbreak, for surveillance purposes, epidemiological analysis, clinical identification of particular strains, vaccine development and monitoring, and understanding the evolution of pathogenic organisms, the step following accurate identification of a microorganism is the more detailed characterization, that is, typing or subtyping the microorganism. The "gold standard" typing method allows typing of all isolates, a high degree of reproducibility, appropriate stability of the targets under investigation, and excellent resolving power (van Belkum et al., 2001). It is also important that typing methods are not too expensive, too complicated, and easily available (van Belkum et al., 2001). The discriminatory power of a typing method can be expressed as a number enabling comparison of diverse typing methods and facilitating choice of the most appropriate method in question of. Nowadays, typing is dominated by molecular methods that have almost completely displaced phenotypic methods. The introduction of molecular techniques in the field of epidemiology has created the discipline of molecular epidemiology. In the field of public health, molecular epidemiology currently mainly rely on PCR-based typing, DNA-fragment-based typing, and DNA-sequence-based typing as depicted in Figure 1.

A methodical exception to the shift from conventional to molecular typing techniques is the classical serotyping of *S. enterica*. Although it has been shown that many Salmonella serotypes comprise strains that have completely different genotypes because of lateral gene transfer of flagella genes (Achtman et al., 2012), implementation of suitable molecular methods is still an issue. Several molecular methods such as PFGE, MLVA, MLST, and HRM have been proposed as alternatives for serotyping but all are hindered by the fact that a one-to-one comparability between serotyping and molecular typing results is impossible. Molecular data show a better correlation to hosts, geographic locations, and antimicrobial resistance than serotyping data, and there is no misleading effect about the disease potential of certain *S. enterica* serovars (Sangal et al., 2010). As a consequence, molecular methods are highly recommended as the superior typing technique for *S. enterica* (Achtmann et al., 2012).

The most widely used molecular typing technique is PFGE. Like AFLP, RFLP, and RAPD, PFGE is a fragment-based technique. The main advantage of fragment-based methods is that no sequence information is needed when performing the analysis. The disadvantage of all fragment-based methods is that band patterns are difficult to compare between laboratories and that reproducibility is low for some of these methods. Nevertheless, PFGE is highly discriminative and shows a good reproducibility (van Belkum et al., 2007) but is limited by interlaboratory comparability, problems of band interpretation, low throughput, sophisticated sample preparation, and relatively high costs. Hence, there is demand for more accurate methods (Cookson et al., 1996). PFGE is also not the optimal method for long-term epidemiological investigations, because it is often too overdiscriminating. Thus, sequence-based methods have an explicit advantage in speed, unambiguous data interpretation, simplicity in the creation of databases, and simplicity of standardization between different laboratories worldwide (MacKenzie et al., 2005). Multilocus sequence typing is a highly discriminatory method, widely used for strain typing. The major disadvantage of this technique is that sequence analysis of seven or more genes is still a time-consuming and costly approach. Furthermore, the submission of data is not automated and, therefore, also time consuming. In conclusion, the length of analysis time impedes the value of PFGE or MLST typing in an outbreak situation because of tremendous political and media pressure and demonstrates beyond all questions the merit of rapid typing methods such as HRM curve profiling (Pietzka et al., 2011).

A clear advancement in sequence-based typing was the introduction of automated *spa* typing via the Internet for *S. aureus* (Harmsen et al., 2003). Owing to this automation, *spa* typing represents a highly effective and rapid typing tool with significant advantages over other typing techniques. In addition, similar *spa* types can be clustered, making this technique suitable for outbreak investigation as well as long-term epidemiological studies (Ruppitsch et al., 2006a; Strommenger et al., 2006).

Besides the ability of a typing method to clearly identify isolates that are involved in an outbreak, the method must accurately differentiate outbreak strains from non-outbreak isolates. Typing methods can be used for a variety of purposes to understand phylogeny (evolution) and

bacterial population genetics, to identify specific strains spreading globally in specific populations and/or in core groups, to identify temporal and geographic changes in strain types as well as the emergence and transmission of individual strains, to establish strain identity/difference in contact tracing or test of cure, to confirm/disprove treatment failures, to resolve medicolegal issues such as sexual abuse, and to confirm presumed epidemiological connections or discriminate isolates of suspected clusters and outbreaks. Finally, such information can be applied to design different public health preventive measures and interventions (Unemo and Dillon, 2011).

WGS has become the ultimate method for the characterization of bacterial isolates as it provides the highest possible resolution in strain typing (i.e., the DNA sequence level) and represents a new paradigm for outbreak investigation and contamination-source tracking. An additional benefit of NGS is the opportunity to extract specific information, such as classical MLST profiles for backward data comparability, the determination of virulence and antibiotic resistance status, as well as the assignment to serogroups as a first-level information, respectively, which is a clear additional benefit of this new technology.

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