

University of Groningen

High Interlaboratory Reproducibility and Accuracy of Next-Generation-Sequencing- Based Bacterial Genotyping in a Ring Trial

Mellmann, Alexander; Andersen, Paal Skytt; Bletz, Stefan; Friedrich, Alexander W.; Kohl, Thomas A.; Lilje, Berit; Niemann, Stefan; Prior, Karola; Rossen, John W.; Harmsen, Dag

Published in:
Journal of Clinical Microbiology

DOI:
[10.1128/JCM.02242-16](https://doi.org/10.1128/JCM.02242-16)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Mellmann, A., Andersen, P. S., Bletz, S., Friedrich, A. W., Kohl, T. A., Lilje, B., Niemann, S., Prior, K., Rossen, J. W., & Harmsen, D. (2017). High Interlaboratory Reproducibility and Accuracy of Next-Generation-Sequencing- Based Bacterial Genotyping in a Ring Trial. *Journal of Clinical Microbiology*, 55(3), 908-913. <https://doi.org/10.1128/JCM.02242-16>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).


Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.



High Interlaboratory Reproducibility and Accuracy of Next-Generation-Sequencing-Based Bacterial Genotyping in a Ring Trial

 Alexander Mellmann,^a Paal Skytt Andersen,^b Stefan Bletz,^a Alexander W. Friedrich,^c Thomas A. Kohl,^{d,e} Berit Lilje,^b Stefan Niemann,^{d,e} Karola Prior,^f John W. Rossen,^c Dag Harmsen^f

Institute of Hygiene, University Hospital Münster, Münster, Germany^a; Department of Microbiology and Infection Control, Statens Serum Institut (SSI), Copenhagen, Denmark^b; Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands^c; Molecular and Experimental Mycobacteriology, Forschungszentrum Borstel, Leibniz-Zentrum für Medizin und Biowissenschaften, Borstel, Germany^d; German Center for Infection Research (DZIF), Borstel, Germany^e; Department of Periodontology and Restorative Dentistry, University Hospital Münster, Münster, Germany^f

ABSTRACT Today, next-generation whole-genome sequencing (WGS) is increasingly used to determine the genetic relationships of bacteria on a nearly whole-genome level for infection control purposes and molecular surveillance. Here, we conducted a multicenter ring trial comprising five laboratories to determine the reproducibility and accuracy of WGS-based typing. The participating laboratories sequenced 20 blind-coded *Staphylococcus aureus* DNA samples using 250-bp paired-end chemistry for library preparation in a single sequencing run on an Illumina MiSeq sequencer. The run acceptance criteria were sequencing outputs >5.6 Gb and Q30 read quality scores of >75%. Subsequently, *spa* typing, multilocus sequence typing (MLST), ribosomal MLST, and core genome MLST (cgMLST) were performed by the participants. Moreover, discrepancies in cgMLST target sequences in comparisons with the included and also published sequence of the quality control strain ATCC 25923 were resolved using Sanger sequencing. All five laboratories fulfilled the run acceptance criteria in a single sequencing run without any repetition. Of the 400 total possible typing results, 394 of the reported *spa* types, sequence types (STs), ribosomal STs (rSTs), and cgMLST cluster types were correct and identical among all laboratories; only six typing results were missing. An analysis of cgMLST allelic profiles corroborated this high reproducibility; only 3 of 183,927 (0.0016%) cgMLST allele calls were wrong. Sanger sequencing confirmed all 12 discrepancies of the ring trial results in comparison with the published sequence of ATCC 25923. In summary, this ring trial demonstrated the high reproducibility and accuracy of current next-generation sequencing-based bacterial typing for molecular surveillance when done with nearly completely locked-down methods.

KEYWORDS whole-genome sequencing, ring trial, interlaboratory reproducibility, cgMLST, molecular subtyping

Today, next-generation sequencing (NGS) is increasingly used to determine the genetic relationships of bacteria on a nearly whole-genome level for infection control purposes and phylogenetic studies. In a shotgun approach, fragmented bacterial DNA is usually sequenced in a highly parallel way, resulting in millions of short reads (up to 400 nucleotides in length) that are either compared to an ideally closely related reference genome (mapping) or are assembled *de novo* for the subsequent extraction of genomic information. Currently, two different approaches, based on single

Received 6 November 2016 **Returned for modification** 30 November 2016 **Accepted** 22 December 2016

Accepted manuscript posted online 4 January 2017

Citation Mellmann A, Andersen PS, Bletz S, Friedrich AW, Kohl TA, Lilje B, Niemann S, Prior K, Rossen JW, Harmsen D. 2017. High interlaboratory reproducibility and accuracy of next-generation-sequencing-based bacterial genotyping in a ring trial. *J Clin Microbiol* 55:908–913. <https://doi.org/10.1128/JCM.02242-16>.

Editor Nathan A. Ledebauer, Medical College of Wisconsin

Copyright © 2017 Mellmann et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Alexander Mellmann, mellmann@uni-muenster.de.

TABLE 1 Summary of sequencing run characteristics and cumulative analysis results from the five participating laboratories

Laboratory designation	Sequencing run characteristics					Sequencing analysis results	
	Cluster density (K/mm ²)	Run output (Gb)	% reads >Q30	Mean read length (bp) ^a	Mean fold coverage (SD ^b , range) ^c	Mean N50	Mean % called cgMLST targets (SD, range)
C1	935	8.6	89.9	225	129 (27, 43–170)	56,620	97.5 (5.7, 73.3–99.6)
C2	715	6.7	92.2	237	106 (17, 76–144)	153,228	99.2 (0.4, 98.0–99.8)
C3	1,297	10.6	87.7	238	169 (54, 29–256)	253,745	98.9 (2.2, 89.7–100)
C4	878	8.2	88.4	221	123 (16, 98–164)	101,873	99.2 (0.4, 98.5–99.9)
C5	1,247	10.8	78.1	180	129 (24, 86–187)	225,594	99.3 (0.3, 98.7–100)
Total mean	1,014	9.0	87.3	220	131 (27, 66–184)	158,212	98.8 (1.8, 91.6–99.9)

^aRead lengths after Illumina base calling and adapter removal.

^bSD, standard deviation.

^cCoverage is calculated for an *S. aureus* genome size of 2.8 Mb.

nucleotide polymorphisms (SNPs) (1, 2) or allelic changes (core genome multilocus sequence typing [cgMLST]) (3–5), are used to extract whole-genome sequencing (WGS) information for subsequently displaying the genotypic relationship.

For continuous infection control surveillance, typing methods should be highly reproducible, ideally generating identical typing results across different laboratories. Previously, we demonstrated that this is the case for *spa* typing that is based on the DNA sequence determination of a repetitive region of the protein A gene (*spa*) of *Staphylococcus aureus* using Sanger sequencing (6). For NGS data, it is known that different sequencing technologies exhibit different error characteristics at the read level (7, 8). Moreover, the analysis pipelines, including assemblers and analytical parameters, can influence the final typing results (7, 9, 10). However, it is unknown how reproducible the overall process of WGS-based bacterial typing is when applied in a multicenter study.

Therefore, we investigated the reproducibility and accuracy of microbial WGS-based typing, employing an international ring trial of five laboratories in three European countries (Denmark, Germany, and The Netherlands).

RESULTS AND DISCUSSION

All five laboratories met the minimum run quality criteria in a single run without repetition (Table 1). Mean sample coverage was 131-fold. However, the coverage per sample varied markedly between 29- and 256-fold, but only samples NGSRT07C1 and NGSRT16C3 exhibited coverages of <75-fold (see Table S1 in the supplemental material). Also, the mean N50 assembly metric parameters differed markedly, whereas the mean percentages of called cgMLST targets were quite even between the laboratories (Table 1). Sample N50 values and percentages of called cgMLST targets were consistently low in samples with <75-fold coverage (see Table S1). All of the reported *spa* types, sequence types (STs), ribosomal STs (rSTs), and cluster types (CTs) were identical (see Table S1). Also, Sanger sequencing-based *spa* typing and BIGSdb revealed identical *spa* types, STs, and rSTs. Only in the two low-coverage samples, the rST, CT, and also the ST for NGSRT16C3, were not assigned. Moreover, in sample NGSRT13C3, the sequence of the 16 repeats containing *spa* type t032 was not determined.

In-depth analysis of the up to 1,861 reported cgMLST genes per sample demonstrated that the majority of isolates shared identical allelic profiles (Fig. 1). A comparison with the controls (NGSRT06-15), which exhibited no deviation, further corroborated this high reproducibility independent from DNA extraction. Samples NGSRT11C1 and NGSRT11C4 varied in one gene (hypothetical protein, SACOL0424), very likely due to a misassembly at the end of the gene. Also, for NGSRT02C1, a wrong allele was called in SACOL2642 (hypothetical protein) due to a low local coverage of 2-fold. These findings are in line with those of a previous study, where an N50 plateau effect for Illumina data was noted above a threshold of 75-fold average coverage (7).

In total, of 183,927 cgMLST allele calls, only 3 (0.0016%) were wrong, resulting in an average of 0.03 wrong alleles per sample. This low error rate does not significantly

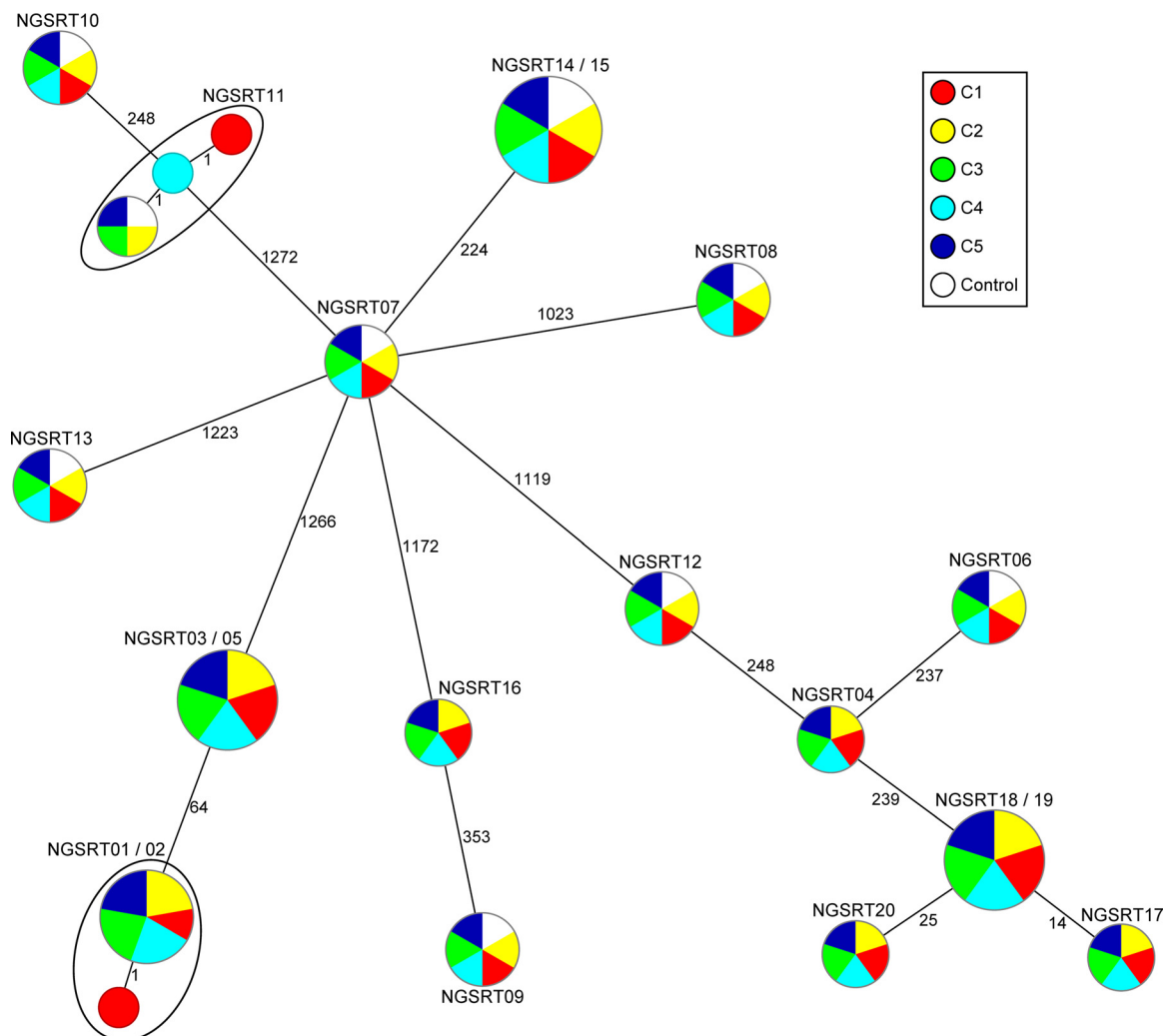


FIG 1 Minimum-spanning tree illustrating the comparison of cgMLST results from the 20 *S. aureus* isolates sent to five laboratories (C1 to C5) in a blinded fashion. Each circle represents a single genotype, i.e., an allelic profile based on up to 1,861 target genes (23) present in the isolates with the “pairwise ignoring missing values” option turned on in the SeqSphere+ software during comparison. The circles are named with the sample ID(s) colored by the participating laboratory, and the sizes are proportional to the number of isolates with an identical genotype. The numbers on connecting lines display the number of differing alleles between the connected genotypes. The control samples colored in white originated from independent cultivations and DNA extractions of samples NGSRT06 to NGSRT15.

affect the ruling in or out of samples during outbreak investigations (11) and ensures high intra- and interlaboratory reproducibilities.

To control the accuracy, the 1,861 cgMLST target sequencing results from sample NGSRT16, i.e., ATCC 25923, which were identical for all five laboratories (Fig. 1), were compared with the published genome sequence of this strain (NZ_CP009361) revealing 12 single nucleotide polymorphisms (SNPs) (see Table S2). Suitable primers for amplifying and subsequent Sanger sequencing were designed for the 12 regions spanning these SNPs (see Table S2) and confirmed that all 12 SNPs were correctly determined during the ring trial. Most likely, the discrepancy with the published sequence can be explained by microevolutionary events that occurred during the freezing, thawing, and repeated cultivation of this strain, which was originally isolated from a clinical specimen in 1945 (12). Similar effects were already previously detected (7).

Our study comprises four limitations. First, we sent only DNA instead of living organisms to the five laboratories, and thereby did not test the influence of DNA extraction methods. The results from the 10 controls analyzed in parallel in the ring trial organizer’s laboratory indicated that this might be of minor importance, as long as high-quality DNA is deployed. Second, we only compared data from one type of

TABLE 2 Characteristics of the 20 human *S. aureus* isolates that were sent as DNA samples to the five participating laboratories in a blinded fashion and used as controls

Sample ID		Spa type (based on Sanger sequencing)	Comment/reference
Ring trial	Original		
NGSRT01	00468	t011	Livestock-associated MRSA
NGSRT02	00551	t011	Livestock-associated MRSA, identical cgMLST genotype as NGSRT01
NGSRT03	01346	t011	Livestock-associated MRSA
NGSRT04	01354	t010	Classical hospital-acquired MRSA
NGSRT05	01360	t011	Livestock-associated MRSA, identical cgMLST genotype as NGSRT03
NGSRT06 ^a	02180	t002	Central European community-acquired PVL ^b -positive MRSA
NGSRT07 ^a	02482	t008	US typical community-acquired PVL-positive MRSA
NGSRT08 ^a	02560	t044	Central European community-acquired PVL-positive MRSA
NGSRT09 ^a	02638	t012	Classical hospital-acquired MRSA
NGSRT10 ^a	02786	t843	<i>mecC</i> -positive MRSA
NGSRT11 ^a	02949	t843	<i>mecC</i> -positive MRSA
NGSRT12 ^a	02994	t003	Classical hospital-acquired MRSA
NGSRT13 ^a	03039	t032	Classical hospital-acquired MRSA
NGSRT14 ^a	COL	t008	MRSA strain COL
NGSRT15 ^a	COL	t008	Duplicate of MRSA reference strain COL
NGSRT16	ATCC 25923	t021	MSSA quality control strain ATCC 25923
NGSRT17	P1	t001	Isolate P1 from reference 23
NGSRT18	P3	t001	Isolate P3 from reference 23
NGSRT19	P4	t001	Isolate P4 from reference 23, identical cgMLST genotype as NGSRT18
NGSRT20	P12	t001	Isolate P12 from reference 23

^aThese samples were separately cultivated, and DNA was extracted and sequenced as controls.

^bPVL, Panton-Valentine leukocidin.

sequencing machine and one type of sequencing chemistry. However, there is evidence from the literature that results from a single laboratory are not significantly biased by the sequencing machine nor by the sequencing chemistry (13, 14). Third, all of the ring trial participants used the same software for analysis. To partially address this issue, we used another tool to verify ST and rST assignment and demonstrate reproducibility across different tools. The Global Microbial Identifier (GMI) wet- and dry-laboratory proficiency test attempts to overcome such limitations, but is challenged by making in-depth comparisons of the heterogeneous results (15). Finally, the accuracy was only determined for cgMLST targets. In accordance with recent practices in public health and clinical microbiology (16), the intergenic regions in particular were not controlled here.

In summary, with the shown high reproducibility and accuracy of WGS-based microbial typing when using a standardized methodology, our study provides the basis for a proficiency testing program, which is one crucial component for ensuring the quality of next-generation sequencing in clinical laboratory practice (17).

MATERIALS AND METHODS

Twenty *Staphylococcus aureus* DNA samples (NGSRT01 to NGSRT20) (Table 2), selected from a diverse collection of isolates (livestock-associated, community-/hospital-acquired methicillin-susceptible and -resistant *S. aureus* from sporadic cases and outbreaks, and a quality control strain), along with duplicates to assess intralaboratory reproducibility, were distributed in a blind-coded manner to the five participating laboratories. DNA samples were prepared using the MagAttract HMW DNA kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions with the addition of 120 U lysostaphin (Sigma, Taufkirchen, Germany) to lyse methicillin-resistant *S. aureus* (MRSA). In addition, the laboratories received a protocol (supplemental material) for performing a single sequencing run on an Illumina MiSeq sequencer using the Nextera XT library preparation kit and the 250-bp paired-end sequencing chemistry version 2 (Illumina, San Diego, CA, USA). Sequencing indices from the Nextera XT index kit were used for multiplexing; participants were free to choose any index combination for the samples. The run acceptance criteria were a sequencing output >5.6 Gb (to achieve an average sequencing coverage of >100-fold for the 20 samples with genome sizes of 2.8 Mb) and a Q30 read quality score of >75%. Otherwise, the sequencing run had to be repeated. SeqSphere⁺ software version 2.4 or higher (Ridom GmbH, Münster, Germany) run on a Microsoft Windows operating system was used with default parameters for quality trimming, *de novo* assembly, and allele calling. Specifically, reads were trimmed at their 5'- and 3'-ends until an average base quality of 30 was reached in a window of 20 bases and subsequently down-sampled to 120-fold coverage. *De novo* assembly was performed using the incorporated Velvet tool version 1.1.04 and a SeqSphere⁺ specific k-mer optimization procedure (18).

SeqSphere⁺ searched the defined genes using BLAST (19) with parameters described previously (20). In addition, the genes were assessed for quality, i.e., the absence of frameshifts and ambiguous nucleotides. A gene was called only if all above-mentioned criteria were met. Thus, determined spa types (10), MLST sequence types (ST) (21), ribosomal MLST types (rST) (22), cgMLST cluster types (CT), and allelic profiles of the 1,861 cgMLST genes (23) were reported to A.M., the ring trial organizer.

In parallel, ST and rST were also determined from the assembly contigs using the BIGSdb system (21, 22, 24). Moreover, spa typing data using Sanger sequencing were available for all isolates (Table 2). Furthermore, 10 strains (NGSRT06 to NGSRT15; in the following named control) were separately cultivated, and DNA was extracted and sequenced in the ring trial organizer's laboratory. For detailed analysis and visualization, a minimum-spanning tree based on the reported cgMLST allelic profiles was constructed using SeqSphere⁺ with the option "pairwise ignoring missing values" turned on. Finally, as we had included as NGSRT16 the well-known quality control strain ATCC 25923 that was recently completely sequenced (12), we determined whether potential discrepancies were due to NGS sequencing errors during the ring trial. Discrepancies that were detected between the published sequence (NZ_CP009361) and the ring trial data from all participants were further analyzed by bidirectional Sanger sequencing from the same DNA that was also sent to the participants. For further confirmatory Sanger sequencing, flanking regions of approximately 250 nucleotides up- and downstream of the detected discrepancies were extracted from the genome sequence, and primers were designed using the NCBI Primer-BLAST service (25). The amplified fragments were purified and Sanger sequenced as described previously (26). The resulting chromatogram files were also analyzed using the SeqSphere⁺ software.

Accession number(s). Raw reads are deposited at European Nucleotide Archive (ENA) under study accession number PRJEB15231.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.02242-16>.

TEXT S1, PDF file, 0.4 MB.

DATA SET S1, XLSX file, 0.8 MB.

DATA SET S2, XLSX file, 0.01 MB.

ACKNOWLEDGMENTS

This study was funded by the European Community's Seventh Framework Program (grant FP7/2007-2013 to A.M., T.K., S.N., and D.H.) under grant no. 278864 in the framework of the European Union Patho-NGen-Trace project and the German Center for Infection Research (DZIF to S.N.).

We thank I. Höfig and A. Schultes (Münster), E. Raangs (Groningen), and J. Zallet and V. Mohr (Borstel) for their help with library preparation, sequencing, and data analysis.

D.H. is one of the developers of the Ridom SeqSphere⁺ software mentioned in the article, which is a development of the company Ridom GmbH (Münster, Germany) that is partially owned by him. The other authors have declared no conflicts of interest.

REFERENCES

- Köser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, Ogilvy-Stuart AL, Hsu LY, Chewapreecha C, Croucher NJ, Harris SR, Sanders M, Enright MC, Dougan G, Bentley SD, Parkhill J, Fraser LJ, Betley JR, Schulz-Trieglaff OB, Smith GP, Peacock SJ. 2012. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. *N Engl J Med* 366:2267–2275. <https://doi.org/10.1056/NEJMoa1109910>.
- Aanensen DM, Feil EJ, Holden MT, Dordel J, Yeats CA, Fedosejev A, Goater R, Castillo-Ramirez S, Corander J, Colijn C, Chlebowicz MA, Schouls L, Heck M, Pluister G, Ruimy R, Kahlmeter G, Ahman J, Matuschek E, Friedrich AW, Parkhill J, Bentley SD, Spratt BG, Grundmann H, European SRL Working Group. 2016. Whole-genome sequencing for routine pathogen surveillance in public health: a population snapshot of invasive *Staphylococcus aureus* in Europe. *mBio* 7:e00444-16.
- Mellmann A, Harmsen D, Cummings CA, Zentz EB, Leopold SR, Rico A, Prior K, Szczepanowski R, Ji Y, Zhang W, McLaughlin SF, Henkhaus JK, Leopold B, Bielaszewska M, Prager R, Brzoska PM, Moore RL, Guenther S, Rothberg JM, Karch H. 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS One* 6:e22751. <https://doi.org/10.1371/journal.pone.0022751>.
- Maiden MC, Jansen van Rensburg MJ, Bray JE, Earle SG, Ford SA, Jolley KA, McCarthy ND. 2013. MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat Rev Microbiol* 11:728–736. <https://doi.org/10.1038/nrmicro3093>.
- Kohl TA, Diel R, Harmsen D, Rothgänger J, Walter KM, Merker M, Weniger T, Niemann S. 2014. Whole-genome-based *Mycobacterium tuberculosis* surveillance: a standardized, portable, and expandable approach. *J Clin Microbiol* 52:2479–2486. <https://doi.org/10.1128/JCM.00567-14>.
- Aires-de-Sousa M, Boye K, de Lencastre H, Deplano A, Enright MC, Etienne J, Friedrich AW, Harmsen D, Holmes A, Huijsdens X, Kearns A, Mellmann A, Meugnier H, Rasheed JK, Spalburg E, Strommenger B, Struelens MJ, Tenover FC, Thomas J, Vogel U, Westh H, Xu J, Witte W. 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. *J Clin Microbiol* 44:619–621. <https://doi.org/10.1128/JCM.44.2.619-621.2006>.
- Jünemann S, Sedlazeck FJ, Prior K, Albersmeier A, John U, Kalinowski J, Mellmann A, Goesmann A, von Haeseler A, Stoye J, Harmsen D. 2013. Updating benchtop sequencing performance comparison. *Nat Biotechnol* 31:294–296. <https://doi.org/10.1038/nbt.2522>.
- Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, Pallen MJ. 2012. Performance comparison of benchtop high-throughput sequencing platforms. *Nat Biotechnol* 30:434–439. <https://doi.org/10.1038/nbt.2198>.
- Jünemann S, Prior K, Albersmeier A, Albaum S, Kalinowski J, Goesmann

- A, Stoye J, Harmsen D. 2014. GABenchToB: a genome assembly benchmark tuned on bacteria and benchtop sequencers. *PLoS One* 9:e107014. <https://doi.org/10.1371/journal.pone.0107014>.
10. Bletz S, Mellmann A, Rothgänger J, Harmsen D. 2015. Ensuring backwards compatibility: traditional genotyping efforts in the era of whole genome sequencing. *Clin Microbiol Infect* 21:347.e1–347.e4. <https://doi.org/10.1016/j.cmi.2014.11.005>.
 11. Mellmann A, Bletz S, Böking T, Kipp F, Becker K, Schultes A, Prior K, Harmsen D. 2016. Real-time genome sequencing of resistant bacteria provides precision infection control in an institutional setting. *J Clin Microbiol* 54:2874–2881. <https://doi.org/10.1128/JCM.00790-16>.
 12. Treangen TJ, Maybank RA, Enke S, Friss MB, Diviak LF, Karaolis DK, Koren S, Ondov B, Phillippy AM, Bergman NH, Rosovitz MJ. 2014. Complete genome sequence of the quality control strain *Staphylococcus aureus* subsp. *aureus* ATCC 25923. *Genome Announc* 2:pii=e011110-14. <https://doi.org/10.1128/genomeA.011110-14>.
 13. Harris SR, Torok ME, Cartwright EJ, Quail MA, Peacock SJ, Parkhill J. 2013. Read and assembly metrics inconsequential for clinical utility of whole-genome sequencing in mapping outbreaks. *Nat Biotechnol* 31:592–594. <https://doi.org/10.1038/nbt.2616>.
 14. Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. 2014. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS One* 9:e104984. <https://doi.org/10.1371/journal.pone.0104984>.
 15. Global Microbial Identifier. 2016. GMI proficiency test report. <http://www.globalmicrobialidentifier.org/Workgroups/GMI-Proficiency-Test-Reports>. Accessed 5 Nov 2016.
 16. Jackson BR, Tarr C, Strain E, Jackson KA, Conrad A, Carleton H, Katz LS, Stroika S, Gould LH, Mody RK, Silk BJ, Beal J, Chen Y, Timme R, Doyle M, Fields A, Wise M, Tillman G, Defibaugh-Chavez S, Kucerova Z, Sabol A, Roache K, Trees E, Simmons M, Wasilenko J, Kubota K, Pouseele H, Klimke W, Besser J, Brown E, Allard M, Gerner-Smidt P. 2016. Implementation of nationwide real-time whole-genome sequencing to enhance listeriosis outbreak detection and investigation. *Clin Infect Dis* 63: 380–386. <https://doi.org/10.1093/cid/ciw242>.
 17. Gargis AS, Kalman L, Berry MW, Bick DP, Dimmock DP, Hambuch T, Lu F, Lyon E, Voelkerding KV, Zehnbaauer BA, Agarwala R, Bennett SF, Chen B, Chin EL, Compton JG, Das S, Farkas DH, Ferber MJ, Funke BH, Furtado MR, Ganova-Raeva LM, Geigenmuller U, Gunselman SJ, Hegde MR, Johnson PL, Kasarskis A, Kulkarni S, Lenk T, Liu CS, Manion M, Manolio TA, Mardis ER, Merker JD, Rajeevan MS, Reese MG, Rehm HL, Simen BB, Yeakley JM, Zook JM, Lubin IM. 2012. Assuring the quality of next-generation sequencing in clinical laboratory practice. *Nat Biotechnol* 30:1033–1036. <https://doi.org/10.1038/nbt.2403>.
 18. Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 18:821–829. <https://doi.org/10.1101/gr.074492.107>.
 19. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215:403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
 20. Ruppitsch W, Pietzka A, Prior K, Bletz S, Fernandez HL, Allerberger F, Harmsen D, Mellmann A. 2015. Defining and evaluating a core genome multilocus sequence typing scheme for whole-genome sequence-based typing of *Listeria monocytogenes*. *J Clin Microbiol* 53:2869–2876. <https://doi.org/10.1128/JCM.01193-15>.
 21. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 38:1008–1015.
 22. Jolley KA, Bliss CM, Bennett JS, Bratcher HB, Brehony C, Colles FM, Wimalaratna H, Harrison OB, Sheppard SK, Cody AJ, Maiden MCJ. 2012. Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. *Microbiology* 158:1005–1015. <https://doi.org/10.1099/mic.0.055459-0>.
 23. Leopold SR, Goering RV, Witten A, Harmsen D, Mellmann A. 2014. Bacterial whole-genome sequencing revisited: portable, scalable, and standardized analysis for typing and detection of virulence and antibiotic resistance genes. *J Clin Microbiol* 52:2365–2370. <https://doi.org/10.1128/JCM.00262-14>.
 24. Jolley KA, Maiden MC. 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595. <https://doi.org/10.1186/1471-2105-11-595>.
 25. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134. <https://doi.org/10.1186/1471-2105-13-134>.
 26. Jenke C, Leopold SR, Weniger T, Rothgänger J, Harmsen D, Karch H, Mellmann A. 2012. Identification of intermediate in evolutionary model of enterohemorrhagic *Escherichia coli* O157. *Emerg Infect Dis* 18: 582–588.