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Fatima Bachir HALIMEH

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The contribution of Molecular Biology in enhancing the knowledge of *Shigella*, *Campylobacter* and *Acinetobacter* in Lebanon

Directeur de thèse **:Walid MOUDANI, Jean-Marc ROLAIN**

Co-encadrement de la thèse : **Rayane RAFEI**

Membres du Jury

M. Jean-Marc Rolain, PU-PH,Université Aix-Marseille	Directeur de thèse
M. Walid Moudani, Professeur,Université Libanaise	Directeur de thèse
Mme. Rayane Rafei, Professeur Assistant,Université Libanaise	Co-directeur de thèse
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Mme. Marie Kempf, PU-PH, Université d'Angers	Rapporteur
M. Ghassan M. Matar, Professeur,Université Americaine au Beyrouth	Examinateur
M. Pierre-Edouard Fournier, Professeur, Université Aix-Marseille	Examinateur
M. Philippe Colson, Professeur, Université Aix-Marseille	Examinateur

Avant-propos

Le format de présentation de cette thèse correspond à une recommandation à la spécialité Pathologie Humaine, Maladies infectieuses, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.
Le candidat est amené à respecter les règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie sur le domaine de cette thèse.

Par ailleurs, la thèse est présentée sur article publié, accepté, ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professor: **Didier Raoult**

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Communications and Workshops

My work has been valued for the moment by the following communications and I had the chance to participate in the following workshops:

Communications:

1- Oral presentation in the 6th Doctoral forum, DSST-Lebanese University (September 27th and September 28th, 2017, Beirut, Lebanon)
2- Poster presentation in the 8th Doctoral forum, DSST-Lebanese University (September 20th, 2018, Beirut, Lebanon)
3- Poster presentation in the 26th Annual Meeting of the Doctoral School in Biology and Health Sciences (May 28th and May 29th, 2018, Marseille, France)

Workshops:

1- Summer School of Medical Mycology- DSST- Lebanese University
2- Bio-Python programming language- DSST- Lebanese University
3- Applicable molecular diagnostics and clinical research- DSST- Lebanese University
4- Workshop in Informatics and their application in healthcare (WIAH 2018)- DSST-Lebanese University
5- Scientific publication: Learning to structure speech in order to publish efficiently- AIX-Marseille University
6- Build, manage, and enhance my thesis project- AIX-Marseille University
7- Functioning efficiently within a work team- AIX-Marseille University
8- Managing Human and work relationships- AIX-Marseille University
9- Quick and efficient reading- AIX-Marseille University
10- Molecular Biology in MEPHI- AIX-Marseille University
11- Hygiene, Safety and Quality in MEPHI- AIX- Marseille University
12- Mass Spectrometry in MEPHI- AIX-Marseille University

Abbreviation list

A. baumannii	*Acinetobacter baumannii*
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Test
C. coli	*Campylobacter coli*
Cg-MLST	Core genome-MLST
CRAB	Carbapenem Resistant *A. baumannii*
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EIEC	Entero Invasive *E. coli*
HAI	Healthcare-Associated Infection
ICU	Intensive Care Unit
MDR	Multi-Drug Resistance
MLST	Multilocus Sequence Typing
NCBI	National Center for Biotechnology Information
NH	Nini Hospital
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
S. boydii	*Shigella boydii*
S. dysenteriae	*Shigella dysenteriae*
S. flexneri	*Shigella flexneri*
S. sonnei	*Shigella sonnei*
SNP	Single Nucleotide Polymorphism
TGH	Tripoli Governmental Hospital
Wg-MLST	Whole Genome- MLST
WGS	Whole Genome Sequencing

WHO	World Health Organization
XDR	Extremely-Drug Resistance
YHC	El Youssef Hospital Center

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Résumé

Bien que la biologie moléculaire soit devenue un pilier dans la plupart des laboratoires de microbiologie clinique dans les pays développés, elle reste encore timide et limitée à un faible nombre des laboratoires dans les pays en voie de développement tels que le Liban. Dans notre premier axe de travail, nous nous sommes penchés sur les problèmes d'identification des Shigelles au Liban en exploitant plusieurs approches phénotypiques et moléculaires sur une collection des isolats identifiés comme Shigelles au nord du Liban. Les résultats hétérogènes des différentes techniques adoptées, soulignent la complexité de différenciation de Shigelles des *Escherichia coli* et la nécessité de la mise en place d'une approche bien validée où le séquençage de génome entier (whole genome sequencing, WGS) est un acteur principal. En deuxième temps, nous avons démontré la puissance du WGS pour déchiffrer simultanément la plupart des mécanismes derrière la multirésistance aux antibiotiques observée chez une souche de *Campylobacter coli* isolée d'un nouveau-né souffrant d'une diarrhée sévère. Finalement, nous avons exploité le rôle du typage moléculaire pour comprendre la nature clonale des souches d'*Acinetobacter baumannii* résistantes aux carbapénèmes, isolées de l'unité de soins intensifs d'un hôpital de soins tertiaires. Le typage nous a assistés d'une part à valider la présence d'une épidémie polyclonale causée par des souches appartenant au ST2 et productrices de carbapénèmases du type OXA-23, et d'autre part à mettre en place des mesures de contrôle opportunes capables d'éradiquer complètement l'épidémie. Nous pouvons ainsi conclure la nécessité d'implanter à large échelle les techniques moléculaires d'identification et de décodage des mécanismes de résistance antimicrobienne dans tous les laboratoires de diagnostic au Liban et le besoin ultime des laboratoires de référence pour le typage et la surveillance des souches afin de faciliter une intégration douce et réussite du séquençage du génome entier. Cette dernière technologie orchestrera certes tous les flux de routine dans un laboratoire de microbiologie clinique dans le futur.

Mots clés : biologie moléculaire, *Shigella* spp., *Campylobacter coli*, *Acinetobacter baumannii*, séquençage du génome entier, identification, résistance, typage**.**

Abstract

Although molecular biology has become a pillar in most clinical microbiology laboratories in developed countries, it is still limited to a small number of laboratories in developing countries such as Lebanon. In our first line of work, we looked at the problems of *Shigella* spp. identification in Lebanon by exploiting several phenotypic and molecular approaches on a collection of isolates identified as *Shigella* spp. in North Lebanon. The heterogeneous results of the different adopted techniques underline the complexity of differentiating *Shigella* spp. from *Escherichia coli* and the need for a well-validated approach in which whole-genome sequencing (WGS) is paramount. Second, we have demonstrated the power of WGS to simultaneously decipher most of the mechanisms behind the multidrug resistance to antibiotics observed in a strain of *Campylobacter coli* isolated from a newborn with severe diarrhea. Finally, we exploited the role of molecular typing to understand the clonal nature of the *Acinetobacter baumannii* strains resistant to carbapenems, isolated from the intensive care unit of a tertiary care hospital. Typing has helped us on the one hand to validate the presence of a polyclonal outbreak caused by OXA-23 carbapenemase-producing ST2 strains, and on the other hand to set up timely control measures capable to fully eradicate the outbreak. Thus, we can conclude the need for large-scale implementation of molecular techniques for the identification and decoding the antimicrobial resistance mechanisms in all diagnostic laboratories in Lebanon as well as the ultimate need for reference laboratories doing strain typing and surveillance, thus facilitating the smooth and successful integration of WGS. Henceforth, this latest technology certainly will orchestrate all routine workflows in a clinical microbiology laboratory.

Keywords: Molecular Biology, *Shigella* spp., *Campylobacter coli*, whole-genome sequencing, identification, resistance, typing.

Introduction

The molecular era has considerably revolutionized the science of Microbiology, by providing fundamental insights into bacteria, viruses, and other microorganisms. Molecular biology, part of medical science, has become increasingly rooted in all microbiological disciplines, including clinical microbiology, and is inspiring a new understanding of the bacterial systematics, the mechanisms and drivers of antimicrobial resistance (AMR), as well as the virulence [1]. In clinical microbiology laboratories, molecular tools have participated in many ways to harness the different tasks and workflows demanded in every-day management of infections [2, 3].

Specific diagnosis of a particular pathogen is one of the primordial tasks of any clinical microbiology laboratory that can impact the tracing of its epidemiological trends and the selection of appropriate treatment. One of the major hurdles hindering such accurate diagnosis is the challengeable identification of some closely related bacteria, such as *Bacillus cereus* complex, *Burkholderia cepacia* complex, *Enterobacter* complex, *Pseudomonas putida* complex, and *Shigella* spp. and *Escherichia coli* (*E. coli*) [4], with negative repercussions on the exact diagnosis of the species involved in the infection, its epidemiology, and the treatment regimen of the patient. For example, *Shigella* spp. has been ranked as the second leading etiology of diarrhea responsible for 212,438 deaths, of which 63,713 are among children under 5 years and 74,402 among persons older than 70 years [5]. While *Shigella* spp. are considered as mandatory notifiable pathogens in many countries, their related bacteria *E. coli* notably entero invasive *E. coli* (EIEC) are not [6], this further emphasizes the critical need for conclusive identification of these allied bacteria. Indeed, the great genetic resemblance between *Shigella* and *E. coli* has been considered for a long time as a subject of debate, and *Shigella* was largely deemed as *E. coli* clones [7]. Recent work has classified *Shigella* as a member of *Escherichia* genera at the same distance of *E. coli* species [8]. This genetic and phenotypic similarities compounded the differentiation between these bacteria using the traditional techniques, and triggered the development of several molecular techniques to resolve this microbiological confusion, and correctly identify *Shigella* spp. [9–11].

Besides identification, fast and accurate assessment of antibiotic susceptibility is an essential step in the clinical laboratories to start effective treatment as soon as possible, prevent the treatment failure, and to track the AMR trends [12]. Molecular biology provides a better understanding of the AMR mechanisms and facilitates the development of several molecular techniques smoothing the detection of genes and mutations associated with antimicrobial

resistance. The polymerase chain reaction (PCR) is the most well-developed molecular technique applied to detect AMR genes, for example, *Streptococcus pneumoniae* macrolide resistance gene (*ermB*), *Staphylococcus aureus* methicillin resistance gene (*mecA*) and *Enterobacteriaceae* beta-lactam resistance genes [13]. However, the range of profiling of antimicrobial resistance mechanisms is often limited by predefined targets and the occurrence of mutations on the primers-specific sequence. Mutations responsible for phenotypic AMR can be characterized by the sequencing of target encoding genes [14]. Furthermore, other molecular techniques for AMR detection are suggested as those relying on DNA hybridization (DNA microarrays and DNA ships) [14]. It is equally important to mention the paramount of applying whole-genome sequencing (WGS) to decipher the entire resistome, giving opportunities to describe novel resistance involved genes, in addition to novel mutations that might be responsible for the resistance phenotyping profile. Although WGS promised to potentially predict AMR by a single assay, there is yet insufficient evidence to replace the phenotypic antimicrobial susceptibility test (AST) by WGS that needs additional standardization and harmonization of available database, as well as quality control metrics [15]. On the other hand, the requirement of expensive WGS platforms and experts in the field of bioinformatics analysis are some of the common limitations, halting the wide integration of WGS, especially in low-income countries [16].

Many pathogens can have an epidemic or even pandemic behavior. Epidemiological investigations and surveillance of serious pathogens through typing techniques are another essential steps of clinical microbiology laboratories. Molecular biology has also made a breakthrough in pathogen typing. Moreover, choosing the convenient molecular typing technique is impacted by the epidemiological purpose and the scale of investigation [17]. In other words, the multi locus sequence typing (MLST) technique is suitable for large scale investigations, including the identification of circulating clones and the surveillance of national and international clone evolution. While pulse-field gel electrophoresis (PFGE) is more dedicated to the small epidemiological scale as the detection of a potential nosocomial outbreak and its reservoir [18]. WGS merges the power of different techniques and can be applied to both large and fine-scale epidemiological investigations, and for different pathogens [17, 18]. While some molecular typing methods are versatile able to apply on different pathogens, others are specific for certain pathogens. Thus, the application of the appropriate molecular typing technique can also be impacted by the investigated pathogen. For instance, insertion sequence IS*6110*-based restriction fragment length polymorphism (RFLP) is the gold standard for

Mycobacterium tuberculosis typing, and SCC*mec* typing and *spa* typing are *Staphylococcus aureus* specific molecular typing techniques [19, 20].

Although clinical microbiological laboratories in developed countries have made tremendous strides in integrating molecular biology in the routine workflow, as well as WGS in the routine typing and surveillance [21], the application of molecular biology remains sparingly used and restricted to a few numbers of laboratories in middle- and low-income countries. The requirement of somewhat expensive supplies forms the major barrier limiting the wide use of molecular techniques in laboratories with scarce resources.

In the present Ph.D. thesis, three different pathogens (*Shigella*, *Campylobacter*, *Acinetobacter*) have been chosen to highlight how molecular biology can engage for a better understanding of these pathogens, that usually cause critical concerns on human public health globally as in Lebanon, especially with the ongoing emergence of AMR. Indeed, different Lebanese studies underlined before the crucial impact of these pathogens in Lebanon and the intricacy of their antimicrobial profiles n [22–26], hence the great need for national surveillance using molecular techniques.

For this, this manuscript is divided into three main Chapters:

Chapter I: involves one review paper and a published article, addressing the challenges in the identification of *Shigella*. In the review paper, we presented the historical, current, and emerging identification methods proposed to untangle the *Shigella*/*E.coli* genetic similarities dilemma, along with a discussion of *Shigella* serotyping techniques. The review will be submitted in the Journal of Microbiological Methods. In the article published in the journal of Acta Microbiologica et Immuniologica Hungarica, we were initially interested in evaluating the identification carried out in Lebanon of the strains identified as *Shigella* spp. and isolated from patients with severe diarrhea and this by comparing several conventional and molecular identification tools. We also assessed the antibiotic resistance of the strains.

In **Chapter II,** we explored the genetic contribution of WGS in explaining the high level of resistance in a *Campylobacter* isolate from newborn suffering from a severe disease. Additionally, we took advantage of the WGS to examine the identity, virulence, and epidemiological background of the strain. The study will be submitted to the journal of Current Microbiology.

Finally, **Chapter III** revealed the potential of the typing approach to control an outbreak caused by carbapenem-resistant *Acinetobacter baumannii* (CRAB), isolated from patients hospitalized in the intensive care unit (ICU). The study is submitted to the journal of Future Microbiology (submitted on 12 April 2020, submission number FMB-2020-0079).

REFERENCES

1. Woese CR (2004) A new biology for a new century. Microbiol Mol Biol Rev MMBR 68:173–186. https://doi.org/10.1128/MMBR.68.2.173-186.2004

2. Medini D, Serruto D, Parkhill J, et al (2008) Microbiology in the post-genomic era. Nat Rev Microbiol 6:419–430. https://doi.org/10.1038/nrmicro1901

3. Bullman S, Lucey B, Sleator RD (2012) Molecular diagnostics: the changing culture of medical microbiology. Bioeng Bugs 3:1–7. https://doi.org/10.4161/bbug.3.1.19011

4. Khot PD, Fisher MA (2013) Novel approach for differentiating Shigella species and Escherichia coli by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 51:3711–3716. https://doi.org/10.1128/JCM.01526-13

5. Baker S, The HC (2018) Recent insights into Shigella. Curr Opin Infect Dis 31:449–454. https://doi.org/10.1097/QCO.0000000000000475

6. van den Beld MJC, Warmelink E, Friedrich AW, et al (2019) Incidence, clinical implications and impact on public health of infections with Shigella spp. and entero-invasive Escherichia coli (EIEC): results of a multicenter cross-sectional study in the Netherlands during 2016-2017. BMC Infect Dis 19:1037. https://doi.org/10.1186/s12879-019-4659-y

7. Pupo GM, Lan R, Reeves PR (2000) Multiple independent origins of Shigella clones of Escherichia coli and convergent evolution of many of their characteristics. Proc Natl Acad Sci U S A 97:10567–10572. https://doi.org/10.1073/pnas.180094797

8. Zuo G, Xu Z, Hao B (2013) Shigella strains are not clones of Escherichia coli but sister species in the genus Escherichia. Genomics Proteomics Bioinformatics 11:61–65. https://doi.org/10.1016/j.gpb.2012.11.002

9. Pavlovic M, Luze A, Konrad R, et al (2011) Development of a duplex real-time PCR for differentiation between E. coli and Shigella spp. J Appl Microbiol 110:1245–1251. https://doi.org/10.1111/j.1365-2672.2011.04973.x

10. Hayford AE, Mammel MK, Lacher DW, Brown EW (2011) Single nucleotide polymorphism (SNP)-based differentiation of Shigella isolates by pyrosequencing. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis 11:1761–1768. https://doi.org/10.1016/j.meegid.2011.07.015

11. Løbersli I, Wester AL, Kristiansen Å, Brandal LT (2016) Molecular Differentiation of Shigella Spp. from Enteroinvasive E. Coli. Eur J Microbiol Immunol 6:197–205. https://doi.org/10.1556/1886.2016.00004

12. Ventola CL (2015) The antibiotic resistance crisis: part 1: causes and threats. P T Peer-

Rev J Formul Manag 40:277–283

13. Yang S, Rothman RE (2004) PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis 4:337–348. https://doi.org/10.1016/S1473-3099(04)01044-8

14. Fluit AC, Visser MR, Schmitz FJ (2001) Molecular detection of antimicrobial resistance. Clin Microbiol Rev 14:836–871, table of contents. https://doi.org/10.1128/CMR.14.4.836-871.2001

15. Ellington MJ, Ekelund O, Aarestrup FM, et al (2017) The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 23:2–22. https://doi.org/10.1016/j.cmi.2016.11.012

16. Wetterstrand KA (2020) DNA Sequencing Costs: Data. In: Genome.gov. https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data. Accessed 21 Jan 2020

17. Rafei R, Kempf M, Eveillard M, et al (2014) Current molecular methods in epidemiological typing of Acinetobacter baumannii. Future Microbiol 9:1179–1194. https://doi.org/10.2217/fmb.14.63

18. Rafei R, Osman M, Dabboussi F, Hamze M (2019) Update on the epidemiological typing methods for Acinetobacter baumannii. Future Microbiol 14:1065–1080. https://doi.org/10.2217/fmb-2019-0134

19. Lakhundi S, Zhang K (2018) Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. Clin Microbiol Rev 31:. https://doi.org/10.1128/CMR.00020-18

20. Ei PW, Aung WW, Lee JS, et al (2016) Molecular Strain Typing of Mycobacterium tuberculosis: a Review of Frequently Used Methods. J Korean Med Sci 31:1673–1683. https://doi.org/10.3346/jkms.2016.31.11.1673

21. CFSAN (2019) GenomeTrakr Network. In: FDA. http://www.fda.gov/food/whole-genome-sequencing-wgs-program/genometrakr-network. Accessed 23 Aug 2019

22. Sabra AH, Araj GF, Kattar MM, et al (2009) Molecular characterization of ESBL-producing Shigella sonnei isolates from patients with bacilliary dysentery in Lebanon. J Infect Dev Ctries 3:300–305. https://doi.org/10.3855/jidc.128

23. Kanafani ZA, Zahreddine N, Tayyar R, et al (2018) Multi-drug resistant Acinetobacter species: a seven-year experience from a tertiary care center in Lebanon. Antimicrob Resist Infect Control 7:9. https://doi.org/10.1186/s13756-017-0297-6

24. Kanj SS, Tayyar R, Shehab M, et al (2018) Increased blaOXA-23-like prevalence in Acinetobacter baumannii at a tertiary care center in Lebanon (2007-2013). J Infect Dev Ctries 12:228–234. https://doi.org/10.3855/jidc.9642

25. Fadlallah SM, Hajj RE, Hage JE, et al (2018) Molecular Epidemiology and Antimicrobial Resistance of Campylobacter coli from Caeca and Carcass of Poultry in Lebanon. Acta Sci Microbiol 1:. https://doi.org/10.31080/ASMI.2018.01.0040

26. Fadlallah SM, Shehab M, Cheaito K, et al (2019) PulseNet Lebanon: An Overview of Its Activities, Outbreak Investigations, and Challenges. Foodborne Pathog Dis 16:498–503. https://doi.org/10.1089/fpd.2018.2581

Chapter I: Challenges in differentiation between *Shigella* spp and *E. coli*.

Introduction:

Shigella is ranked as the second bacterial etiological cause of diarrhea worldwide, and as the first causative agent in developing countries [1]. Based on consecutive research to understand the phenotypic and genotypic similarity between *Shigella* spp. and *E. coli* and unveil the true taxonomic position of *Shigella* spp, a recent study demonstrates that *Shigella* spp. are not *E. coli* clones as widely acknowledged, but an *Escherichia* species, as the same distance of *E. coli* [2]. According to the genetic closeness between *Shigella* spp. and *E. coli* notably EIEC, the phenotypic differentiation remains restricted to a few biochemical tests [3]. This phenotypic and genetic closeness makes the differentiation more challenging, especially in developing countries with limited resources. Such differentiation is a watershed step to reliably draw the epidemiological picture of *Shigella*, estimate the AMR trends, and select the appropriate therapeutic regimen. Additionally, serotyping is another cornerstone to identify *Shigella* spp., track the potential emersion of any novel serotype, investigate outbreaks, and critically appraise implemented policies for vaccine development and disease containment.

In this chapter, we aimed to shed light on the differentiation dilemma of these two allied bacteria. The chapter involves a review paper and a published study.

In the review paper entitled **"Historical, current, and emerging tools for identification and serotyping of *Shigella*"**, we provide an updated view on the current and emerging *Shigella* identification and serotyping techniques from phenotypic to molecular techniques. The review also discusses their resolution and their discriminatory power to differentiate between *Shigella* spp. and *E. coli*. Finally, we supported that the WGS is a promising technology that allows accurate identification of *Shigella* at species level through many approaches as those based on single nucleotide polymorphisms (SNP) or Core genome-MLST (Cg-MLST). Besides, WGS allows maintaining backward compatibility with the conventional serotyping scheme and this by *in silico* serotyping based on WGS data.

The second part of the chapter is an article entitled **"Challenges in identification of enteroinvasive *Escherichia coli* and *Shigella* spp. in Lebanon"**. In this article, we assessed the accuracy of the routine identification of a collection of clinical isolates identified as *Shigella* spp. by the biochemical gallery Api 20E, and this by combining several phenotypic (Api 20E, MALDI-TOF, and conventional agglutination tests) and molecular (*ipaH* and *lacY* PCR and *gyrB* gene sequencing) techniques. We had also the opportunity to characterize the AMR mechanisms against beta-lactam harbored by our isolates. Our results demonstrated the heterogeneous identity of the tested collection that encompassed EIEC, *E. coli*, *Shigella*, and inactive *E. coli* isolates. Generally, the used methods yielded discordant identification results

and answered differently the tackling question. While each method has its own strengths, WGS appeared here the optimal method (although not applied herein for logistical reasons) for such a dilemma and a key step to choose the most appropriate cost-effective identification method to be integrated into the routine workflow in the middle and low-income countries. Although this study was not able to unveil the true identity of the isolates, it brings into sharp focus the importance of accurate identification of *Shigella* and *E. coli* isolates keeping in mind that these strains expressed resistance to beta-lactams and harbored the widespread beta-lactamases *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-15}}$, and *bla*$_{\text{CTX-M-3}}$.

REFERENCES

1. Baker S, The HC (2018) Recent insights into *Shigella*. Curr Opin Infect Dis 31:449–454. https://doi.org/10.1097/QCO.0000000000000475

2. Zuo G, Xu Z, Hao B (2013) *Shigella* strains are not clones of *Escherichia coli* but sister species in the genus *Escherichia*. Genomics Proteomics Bioinformatics 11:61–65. https://doi.org/10.1016/j.gpb.2012.11.002

3. van der Ploeg CA, Viñas MR, Terragno R, et al (2010) Laboratory Protocol: Serotyping of *Shigella* spp. Geneva

Review paper: Historical, current, and emerging tools for identification and serotyping of Shigella.

Fatima Bachir Halimeh[1,2], Rayane Rafei[1], Marwan Osman[1], Seydina Diene[2], Fouad Dabboussi[1], Jean-Marc Rolain[2], Monzer Hamze[1*]

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[1]Laboratoire Microbiologie Santé et Environnement (LMSE), Ecole Doctorale des Sciences et de Technologie, Faculté de Santé Publique, Université Libanaise, Tripoli, Liban.

[2]Aix-Marseille Univ., MEPHI, IRD, APHM, IHU-Méditerranée Infection, 19-21 boulevard Jean Moulin, 13385 Marseille CEDEX 05, France.

* Corresponding author

Monzer Hamze: mhamze@monzerhamze.com

Abstract

Shigella is a serious foodborne disease causative agent, with four species and 54 serotypes. Identification at both species and serotype levels is a paramount task in microbiological laboratories. Nevertheless, the genetic similarity between *Shigella* spp. and *Escherichia coli* challenges such as correct identification and serotyping of *Shigella* spp., with subsequent negative repercussions to the surveillance and epidemiological investigations as well as to the selection of appropriate treatment. Throughout history, a bunch of techniques has been developed from phenotypic passing through single or multilocus molecular techniques to Whole Genome Sequencing (WGS). To facilitate the selection of the most relevant method, we provide herein the reader with a global overview of historical and emerging identification and serotyping techniques with a special focus on WGS-based approaches. This review supports the excellent discriminatory power of WGS to elucidate the true epidemiology of *Shigella* spp, disclose novel promising genomic targets for surveillance methods, and validate previous well-established methods.

Keywords: *Shigella* spp. *Escherichia coli*, identification, serotyping, Whole Genome Sequencing.

Highlights:

- The genetic and phenotypic closeness of *Shigella* and *E. coli* is a diagnosis issue
- Many molecular algorithms are supposed to differentiate between EIEC and *Shigella*
- These algorithms need more validation for complete integration in the routine
- Genoserotyping is a promising alternative for standard *Shigella* serotyping method
- WGS is the future gold standard method for both *Shigella* diagnosis and surveillance

Overview:

Shigella has been known for a long time by its clinical entity "bacillary dysentery", even before its identification by Kiyoshi Shiga as the causative agent during a severe Japanese outbreak [1]. Nowadays, it ranks the second leading etiology of diarrhea mortality responsible annually for approximately 212,438 deaths, of which 63,713 are among younger children than 5 years and 74,402 among persons older than 70 years [2]. Although primary the disease of developing countries, *Shigella* infection or shigellosis remains a public health issue with nearly 500,000 illnesses in the United States annually [3]. *Shigella* is transmitted through the oral-fecal pathway with a low infectious dose where 10 to 100 organisms can introduce the disease [4]. The shigellosis is a non-systematic, enteric, and acute infection characterized by colonic epithelium destruction responsible for bloody diarrhea, sometimes accompanied by mucus, abdominal pain, and fever [5]. Occasionally, *Shigella* can lead to invasive infections such as meningitis, osteomyelitis, spleen abscess, and sepsis, occurring mainly in malnourished and HIV patients [6].

Shigella species are intracellular Gram-negative, facultative aerobic, and nonsporulating bacilli belonging to the *Enterobacteriaceae* family [7]. Although acknowledging the close relatedness with *Escherichia coli,* the new genus *Shigella* with its four species were formally described in 1940 following the recommendations of Ewing [8]. Since their discovery, several studies attempted to accurately classify *Shigella* spp. within the *Enterobacteriaceae* family. In 1982, based on 192 morphological, biochemical, and phenotypic characters, Dodd and Jones, have determined that *Shigella* spp. fell into a major distinct cluster more closely related to *Yersinia* and *Proteus/Providencia* species than *E. coli* [9]. This phenotypic classification strengthens the traditional separation of *Shigella* as a separate genus. However, with the upcoming of the molecular era, many ambiguities have arisen about the precise taxonomical position of *Shigella* and the dilemma of relatedness with *E. coli* remains thorny. *Shigella* spp. and *E. coli* appeared as "one genetically species" with DNA-DNA hybridization (DDH) experiments presenting 80-90% identity [10]. After that, the sequencing analysis of eight housekeeping genes grouped *Shigella* into three major clusters and a limited number of outliers that have found to be evolved independently from multiple non-pathogenic *E. coli* ancestors [11]. Accordingly, the results of the phylogenetic relationships driven from the analysis of three plasmidic gene sequences (*ipgD*, *mxiC*, and *mxiA*) harbored by the invasion plasmid (pINV) are largely consistent with the previous observations based on chromosomal genes [12]. Therefore, *Shigella* spp. are considered as *E. coli* clones [11]. Even, with the availability of complete sequenced genomes, the inclusion of either more

housekeeping genes in analysis or the whole set of conserved genes (also known as the core genome) confirmed previous findings that *Shigella* spp. are intermixed with *E. coli* [13,14]. Nowadays, there is a consensus that *Shigella* belonged to the *E. coli* species, but the nomenclature has been kept for historical and medical reasons [15]. However, a recent whole genome-based, alignment-free, and parameter-free method also called CVTree, revealed that the four species of the *Shigella* genus are not clones of *E. coli*, but members of the *Escherichia* genus at the same footing with *E. coli* [15–17].

Although challengeable in clinical microbiology laboratories, differentiating *Shigella* from *E. coli* can be guided by many distinctive morphological features. Indeed, more than 80% of *E. coli* strains are motile, able to decarboxylate lysine and ferment many sugars, indole positive, and produce gas from D-glucose. Nevertheless, *Shigella* are non-motile, unable to decarboxylate lysine, do not produce acid from salicin nor hydrolyze esculin, ferment few sugars, and do not produce gas from D-glucose, except *Shigella flexneri* serotype 6 and *Shigella dysenteriae* 3. Additionally, *Shigella sonnei* strains can ferment lactose slowly and be mucate positive [12,18]. However, a group of *E. coli* variants called "inactive *E. coli*", which includes the enteroinvasive *E. coli* (EIEC) pathovar, shares with *Shigella* some biochemical properties among others negativity to lactose, immobility, and absence of gas production [19]. Comparing with *Shigella* spp., EIEC strains cause the same disease symptoms and harbor similar invasion plasmid (pINV) but are generally less virulent with a higher infectious dose [20]. EIEC can be differentiated from *Shigella* only by a very limited number of tests, which include motility, mucate, and salicin fermentation, esculin hydrolysis, the combined positivity of indole production and gas formation form D-glucose, and acetate utilization. EIEC isolates may be positive for one or more of the tests but *Shigella* are generally negative [21,22]. Furthermore, a key combining biochemical, physiological, and serological features was designed for the daily identification of EIEC, *E. coli,* and *Shigella* in diagnostic laboratories [22].

Amid the technologic evolution, an accurate technique able to differentiate between *E. coli* and *Shigella* spp. continues to be a badly need. As alluded above, the genetic and phenotypic relatedness between *Shigella* and *E. coli* notably EIEC presents a significant diagnostic challenge. This distinction is paramount since *Shigella* is a mandatory notifiable disease in most countries whereas EIEC is not [23]. The correct differentiation will also help to well elucidate their epidemiology and their trends of developing antimicrobial resistance, as well as to better address the treatment regimen due to different antimicrobial susceptibility profiles [24]. Additionally, quite pronounced is the differentiation between *Shigella* spp. in order to track their epidemiological behaviors as each species draws its unique epidemiology;

for example, while *S. flexneri* prevailed in low- and middle-income countries, *S. sonnei* dominated in high-income countries [6]. Besides, the correct identification at serotype level is another cornerstone to determine the spatial-temporal distribution of circulating serotypes, understand the differences in disease burden across countries, track the potential emersion of any novel serotype, investigate outbreaks, and critically appraise implemented policies for vaccine development and disease containment. Herein, we will discuss both, phenotypic and molecular identification techniques used to differentiate *Shigella* from EIEC and identify *Shigella* at the species level (Table 1, Figure 1). Our review also highlights the inherent loopholes in the phenotypical serotyping strategy of *Shigella* and recaps the proposed molecular serotyping alternatives.

1. Phenotypic identification techniques:

1.1 Biochemical test systems:

Commercial biochemical identification systems are based on one of five different technologies or a combination thereof: pH-based reactions, utilization of carbon sources, enzyme-based reactions, visual detection of bacterial growth, or detection of volatile or nonvolatile fatty acids [25] The tests dedicated for *Enterobacteriaceae* identification are multiple and can be categorized into manual as API 20E, and RapiD 20 E (BioMérieux, Marcy-l'Étoile, France), RapID ONE and Micro-ID (Remel, San Diego, California, United States) and automated ones as BD Phoenix 100 ID/AST system NID panel (Becton Dickinson, New Jersey, United States), Vitek 2 (BioMérieux), and MicroScan Neg ID Type 2 (Beckman Coulter, California, United States). Focusing on their efficiency in identifying *Shigella*, Api 20E largely accepted in the last decades in the clinical microbiology laboratories failed to identify 3% to 10% of *Shigella* strains [25–27], while BD phoenix misidentified nearly 17% of *Shigella* isolates and defined them as *E. coli* [28–30]. Vitek2 repeatedly misidentified a commensal inactive *E. coli* as *S. sonnei* [31]. However, the reliability of these evaluation studies is equivocal where the adopted gold standard (as conventional or commercial biochemical methods) was generally questionable to its ability to separate *E. coli* and *Shigella* [29,32].

1.2 Serotyping:

The four *Shigella* species (subgroup) are divided into serotypes and subserotypes (except *S. sonnei*) based on their O antigen: *S. dysenteriae* (subgroup A) has 15 serotypes, *S. flexneri* (subgroup B) has 18 serotypes, *Shigella boydii* (subgroup C) has 20 serotypes and *S. sonnei* (subgroup D) with a single serotype [4]. Notably, a confirmed identification of *Shigella* spp. must be based on both serological and biochemical profiles [33]. Traditionally, serotyping

was performed using *in house* or commercial antisera to LPS O-antigen that are divided into polyvalent and monovalent antisera. The polyvalent antisera contain antibodies for multiple *Shigella* serotypes and can subsequently determine *Shigella* subgroups, while the monovalent antisera contain serotype-specific antibodies [33]. Among the most common commercialized *Shigella* serotyping antisera kits are Wellcolex™ Color *Shigella* Kit (Thermo Fisher Scientific Inc., Massachusetts, United States) and Vision™ Polyvalent *Shigella* Antisera (ProLab Diagnostics Inc., Ontario, Canada) providing polyvalent antisera. Meanwhile, some companies provide both polyvalent and monovalent antisera as BioRad Laboratories Inc., (California, United States), Deben Diagnostics Ltd., (Ransomes industrial estate, United kingdom), and Denka Seiken Co., Ltd. (Tokyo, Japan). While serotyping was admittedly regarded as the gold standard for *Shigella* species identification [30], it is considered laborious, time-consuming, and not practical for a large number of samples. Besides, additional issues lessen the usefulness of such an approach. First, many intra- and inter-species cross-reactions are observed and commercial antisera are ideally 91% accurate [34]. During a cohort test, 28% of *S. sonnei* isolates were misidentified by conventional serotyping techniques, and additional tests such as PCR of *ipaH* and *lacY* genes or repeated serotyping are used to resolve this discrepancy [30]. Indeed, inherent similarities between *E. coli* and *Shigella* O-antigens hinder the reliability of serotyping. Of 34 distinct O antigens identified in *Shigella*, 21 are identical or very similar to those described in *E. coli* [18]: EIEC O112ac similar or identical to *S. dysenteriae* 2/*S. boydii* 15/*S. boydii* 1, EIEC O124 to *S. dysenteriae* 3/provisional *Shigella* serovar 3615.53, EIEC O136 to *S. dysenteriae* 3/*S. boydii* 1, EIEC O143 to *S. boydii* 8, EIEC O152 to provisional *Shigella* serovar 3341:55, EIEC O135 to *S. flexneri* and EIEC O164 to *S. dysenteriae* 3. Second is the problem of occasional provisional *Shigella* serovars biochemically indistinguishable from *Shigella* spp., but failing to agglutinate with standard commercial antisera [35,36]. Such observations may be due to morphologic transition from smooth to untypable rough strains without O antigens, which accounts for 6 to 10% of annual *Shigella* in the USA [34]. The presence of capsular antigens may also prevent *Shigella* strains to react with the antisera [33]. Additionally, the emergence of such novel and atypical serotypes able to escape host immunity responses can also be explained by serotype conversion phenomena mediated by either temperate bacteriophages or plasmids carrying serotype encoding genetic elements [37–39]. The diagnosis techniques for these non-serotypeable *Shigella* are discussed after in this present. Third, reciprocal connections between biochemical features, serotypes, and phylogenetic relationships do not exist necessarily. Indeed, the phenotypic variability observed within a particular serotype raises

when increasing the testing of membered isolates on the one hand and the presence of serotypes that are genetically and serologically related but unrelated phylogenetically falling into distinct *Shigella* clades convoluted further the usefulness of serotyping on the other [36,37].

1.3 MALDI-TOF MS:

Matrix-Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS) is recently reckoned as a rapid, cost-effective, high throughput and reliable microbial identification tool with wider applicability to a large spectrum of microorganisms [40]. Notwithstanding this versatility, conventional MALDI-TOF assays using MALDI-TOF Biotyper (Bruker Daltonics, Bremen, Germany), and VITEK 2 MS systems (bioMérieux, Marcy l'Etoile, France), failed to distinguish *Shigella* spp. from *E. coli* due to the high degree of similarity between their spectra [30,41]. However, studies suggesting the use of a specialized automated algorithm (ClinProTools) or customized reference library reflecting the genetic diversity of *Shigella* and *E. coli* revealed an outperformance over routine MALDI-TOF assays by enabling accurate discrimination between *E. coli* and *Shigella* with misidentification rates reaching up approximately 3% [30,42]. Recently, an approach merging the biochemical method and the MALDI-TOF assays seems to be interesting. By adding a short term incubation in a high-lactose fluid medium before MALDI-TOF analysis, Ling *et al.*, identified seven novel differential MS peaks serving as biomarkers to reliably identify these allied bacteria with nearly 98% accuracy [43].

2. Molecular identification techniques:

2.1. PCR Techniques:

In the last decades, several PCR-based identification techniques were suggested to differentiate between *Shigella* spp., *E. coli*, and EIEC [44,45]. However, the difficulty facing the PCR development dwells in the selection of appropriate targets allowing an accurate delineation of the different classes wherein some PCR were unable to separate *Shigella* from EIEC [46,47]. Generally, PCR identification schemes for *Shigella* often target plasmidic virulence genes as *ipaH*, *virA*, *ial*, *she*, and *tuf*, which are vulnerable to horizontal gene transfers leading to false positive and negative results [48]. One of the main gene targets commonly integrated into PCR schemes is *ipaH*, a multicopy virulence factor encoding gene located on both the chromosome and the large invasion plasmid pINV exclusively found in *Shigella* and EIEC isolate [48,49]. Recently, *ipaH* amplification was used as the first step in two different algorithms proposed for the *Shigella* identification, to differentiate between the *Shigella*/EIEC (*ipaH* +) and non-invasive *E. coli* (*ipaH* -). The culture-dependent algorithm was followed by profiling phenotypical, biochemical, and serological features; whereas the molecular algorithm

targeted additionally the *wzx* genes of *S. sonnei* phase I, *S. flexneri* serotype 1-5, *S. flexneri* 6, and *S. dysenteriae* serotype 1 [36]. After discrepancy analysis with Whole-Genome Sequencing (WGS), the culture-dependent algorithm succeeded to identify 100% of *S. dysenteriae*, *S. sonnei* and non-invasive *E. coli* isolates, but only 85% of *S. flexneri*, and 93% of *S. boydii* and EIEC. With regards to the molecular algorithm, while it fully identified all targeted species or serotypes, it could not specifically detect the *ipaH* positive serotypes with none assessed *wzx* and binned them into a single group as either EIEC, *S. boydii*, *S. sonnei* phase II, or *S. dysenteriae* serotype 2-15 [36].

In order to delineate *Shigella* from EIEC, one PCR scheme amplifies, in addition to *ipaH* specific to EIEC and *Shigella*, a lactose permease encoding gene (*lacY*) present in *E. coli* including EIEC [45]. This last scheme enabled the differentiation of EIEC O121 and O124 groups and *Shigella* but was unable to fairly classify EIEC O164 group. Another scheme delineates *Shigella* from *E. coli* including EIEC by targeting the β-glucuronidase-encoding gene (*uidA*) commonly found in both *E. coli* and *Shigella* spp. and *lacY* specifically observed in *E. coli* strains [44]. However, the accuracy of this PCR appeared not as excepted, while it correctly identified *in silico* 100% of *S. sonnei*, it failed to define 8% of *S. flexneri*, 14% of *S. boydii*, 20% of *S. dysenteriae*, 23% of non-invasive *E. coli*, and 38% of EIEC isolates [50]. Moreover, the utility of *lacY* can be questioned by the fact that even *S. flexneri* and *S. boydii* lack the *lac* genes (*Y*, *A*, and *Z*), other *Shigella* spp. possess some *lac* genes. *S. dysenteriae* has *lacA* and *lacY* genes and *S. sonnei* has all the *lac* genes but they are unable to ferment lactose due to the lack of the permease activity [51]. Besides, after 4h enrichment of the sample in a growth medium, a conventional pentaplex PCR could identify *Shigella* at genus level and differentiate between *S. flexneri*, *S. sonnei* and *S. dysenteriae* by amplifying specific targets *invC*, *rfc*, *wbgZ*, and *rfpB* respectively together with an internal control (*ompA*) [52]. Interestingly, a new proposed phylogenomic-based multiplex PCR assay by Sahl *et al.*, was able to both identify unknown *Shigella* isolates and classify them into appropriate phylogenetic clades [53]. Meanwhile, when primers are tested on a considerable genetically diverse collection, they could not phylogenetically differentiate *Shigella* [50]. To override the issue of targeting plasmidic virulence genes, Kim *et al.*, designed primers targeting novel genetic markers identified through comparative genomics that are able to differentiate *Shigella* from diarrheagenic *E. coli* including invasive *E. coli* and identify the four *Shigella* spp. [48]. Additional steps can be added to PCR as Restriction Fragment Length Polymorphism (PCR-RFLP) [54], immunocapturing technology [55], to increase either the sensitivity and/or specificity of detection. However, some of these methods are relatively expensive, and difficult

and require special equipment hindering thereby their applications as diagnostic or epidemiological tools.

2.2 Single locus sequence-based identification techniques:

***16S rDNA* gene sequencing:**

Although representing 0.1% of the coding part of a microbial genome, *16S rDNA* gene sequencing has been reckoned as a highly useful tool in bacterial classification and has been widely used to provide genus and species identification for isolates. However, its usefulness is impaired by its low discriminatory power and its poor resolution to distinguish between closely related bacteria [56,57]. The reported *16S rDNA* sequence similarities between *E. coli* and *Shigella* spp. exceed 99%, reaching up to 99.8% with *S. flexneri*, 99.9% with *S. sonnei*, and 99.7% with *S. boydii* [57]. For this, *16S rDNA* sequencing is not considered a reliable tool for differentiating between *E. coli* and *Shigella* spp., wherein they were intermingled together in the *16S rDNA*-based tree. Using Sanger sequencing, only 26.7% of the *E. coli* strains were correctly identified, compared to 33.3% as *S. sonnei* and 40% as *S. dysenteriae* [58]. The species finder, a web-based tool for prokaryotic species identification based on the similarity of *16S rDNA* sequences (https://cge.cbs.dtu.dk/services/SpeciesFinder/) with the known reference sequences available at the center of genomic epidemiology, revealed the poorest performance against KmerFinder (another *in silico* tool, explained later in the review) and *gyrB* sequence analysis, wherein only 74% of non-serotypeable *Shigella* were reliably identified to the species level [57].

***rpoB* gene sequencing:**

Being a single copy protein-encoding housekeeping gene, *rpoB* can be more advantageous than the *16S rDNA* sequence in microbial identification. While *rpoB* is deemed as a high-resolution marker able to reveal molecular variation down to the population level, it has an overlapping similarity between closely related isolates as *Shigella* and *E. coli* [59]. The *rpoB* sequence similarities between *E. coli* and *Shigella* spp. exceed 93% [59], reaching up to 99.8%. with *S. flexneri*, 99.4% with *S. sonnei*, and 99.78% with *S. boydii*. However, Ragupathi *et al.*, revealed that *rpoB*, as well as another housekeeping gene malate–lactate dehydrogenase (*mdh*), accurately identified *Shigella* and different *E. coli* virotypes [51].

***gyrB* gene sequencing:**

Compared to *16S rDNA*, the *gyrB* encoding the ß subunit protein of the DNA gyrase (Topoisomerase Type II) seems to have a greater evolutionary divergence for the bacteria with an ability to distinguish between the closely related species. With regards to *E. coli* and *Shigella* spp, the *gyrB* similarity percentages between *E. coli* and either *S. sonnei, S. flexneri*, or *S. boydii*

were 98.1, 97.8 and 98%, respectively; being lower than those obtained with *16S rDNA* analysis hints seemingly for the potential accurateness of *gyrB* gene sequence analysis [60]. Many studies revealed an outperformance of *gyrB* over *16S rDNA* sequencing [57,61], where the identification results of *gyrB* sequencing were highly congruent to the Kmerfinder tool with 100% identification of non-serotypeable isolates at the species level [57,62].

2.3 Whole-genome sequencing:

Cumulative data generated from many genetic and intergenic regions could unravel the real identity of an isolate. In this line, decoding the bacterial genome via WGS is a very promising technology, particularly with its precipitously decreasing cost, to replace the conventional microbial diagnostic workflow and become a public health resource for global surveillance [63]. WGS can be followed by multiple analyses to identify, serotype, classify *Shigella* spp., and even understand their pathogenesis. Numerous enticing WGS-based approaches were assessed to their ability to differentiate *Shigella* and *E. coli* including K-mers, whole-genome Single Nucleotide Polymorphism (SNP), and Average Nucleotide Identity (ANI) [50,64,65]. Notably, the common limitation hindering the wide integration of WGS especially in low-income countries is the requirement of the WGS platforms and experts in the bioinformatic analysis.

K-mers-based approaches:

K-mers-based species identification tools (as Kmerfinder the online tool available at https://cge.cbs.dtu.dk/services/KmerFinder/) split the WGS data of unknown isolate into relatively short oligomers of a defined length k, then compared the resulting content of k-mers to a set of k-mers derived from a collection of reference genomes [57,64,66]. The similarity between the two query and reference sets was expressed as a percentage value indicating the portion of common kmers. The kmer identification concordantly predicted 98.4% of 1982 *Shigella* and *E. coli* isolates as that obtained with the traditional biochemistry and serology scheme. The 25 discrepant results revealed either the superiority of kmer approach over the traditional scheme when unfunctional O antigen biosynthesis genes in *S. flexneri* could traditionally misidentify them as *S. boydii*, or the inferiority of kmer notably for 10 EIEC isolates misidentified as *S. flexneri* or *S. boydii* by the kmer derived identification [64].

SNP based approaches:

SNP based approaches catch only informative genetic signatures in both gene-encoding and intergenic regions, omitting thus the inclusion of genetically conserved meaningless data [67]. Therefore, SNP, considered generally as stable and reproducible molecular markers, can provide additional strain differentiation at a meticulous level ultimately important for outbreak

investigation and surveillance strategies of important pathogens as *Shigella* spp. [50,68]. Beyond typing scope, SNP analysis can also serve in drawing the true phylogeny of *Shigella* spp. and deciphering their enigmatic relations with EIEC [50]. Additionally, the obtained SNPs are valuable markers for the development of rapid, accurate, and discriminative diagnostic methods [68,69]. Based on *in silico* analysis of eight *Shigella* genomes, 24 informative SNPs selected from nine genes (*gapA*, *lpxC*, *sanA*, *thrB*, *yaaH*, *ybaP*, *ygaZ*, *yhbO*, and *ynhA*) were found useful in identifying *Shigella* spp. as well as providing some resolving power among individual strains within the same species [69]. When analyzing a comprehensive collection of *Shigella* and EIEC, Pettengill *et al.*, identified a panel of 254 SNP markers able to accurately identify and type EIEC and *Shigella* spp. from WGS data [50].

ANI-based approaches:

The Average Nucleotide Identity (ANI) between two genomes has been suggested as a valid alternative to the wet-lab DDH methods for species delineation where genomes can be defined as members of the same species if sharing ≥95-96% ANI [70]. Various ANI-based approaches and software are nowadays available to *in silico* compare the genomes [65]. To reduce the high computational methods of ANI-based approaches, a novel method known as the Whole Genome Parameter (WGP) was proposed for the delineation of bacterial genomes using four statistical parameters calculated from numerical representations of whole bacterial genomes (phase signal and cumulated phase signal) [65]. However, when these aforementioned delineation methods (the ANI-based tools and WGP) are tested to their ability to delineate *Shigella* spp. from *E. coli*, the majority including the WGP failed thus mirroring the inability of traditional DDH to separate this group [65]. Notably, the GGDC web tool with the ANI-f1 formula, one of the tested ANI-based delineation tools, showed some power in differentiating *E. coli* from *Shigella* spp. [65].

Extended MLST schemes:

Multi Locus Sequence Typing (MLST) is a sequence-based genotyping technique based on the sequencing of many housekeeping genes. Three MLST schemes developed originally for *E. coli* have also been applied to *Shigella*: the Achtman scheme includes seven housekeeping genes (*adk, fumC, gyrB, icd, mdh recA* and *purA*), while the Pasteur scheme contains eight genes (*dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB*, and *uidA*), and the Whitmann scheme 15 genes (*arcA*, *aroE*, *aspC*, *clpX*, *cyaA*, *dnaG*, *fadD*, *grpE*, *icdA*, *lysP*, *mdh*, *mtlD*, *mutS*, *rpoS*, *uidA*) [71–73]. Even that sequence types (ST) were assigned by MLST schemes regardless of species identity either *E. coli* or *Shigella*, categorizing isolates into ST seems to mirror the *Shigella* classification [64] where the majority of isolates within the same species

had closely related STs belonging to the same clonal complexes (CCs). Chattaway *et al.*, suggested the combined use of kmer and MLST to differentiate *E. coli* from *Shigella* [64]. However, some CCs can encompass many species as the case of CC288 membered by both *S. boydii* and *S. dysenteriae* isolates [64].

Thanks to the advent of WGS, the legacy MLST schemes with usually seven genes can be extended to encompass more *loci* distributed over the chromosome (WgMLST or Whole genome MLST) or conserved *loci* shared among most of the isolates of the same species (cgMLST or core genome MLST). Undoubtedly, these new MLST facets give a deep genomic insight and high resolution than the conventional MLST, especially for closely related bacteria [67,74,75]. For *Shigella* and *E. coli*, the same cgMLST and wgMLST schemes available at the publicly accessible Enterobase database can be used (http://enterobase.warwick.ac.uk/species/index/ecoli) for the two species. Besides their promises in typing, the cgMLST technique demonstrates its capability in resolving the discrepancies raised between the culture-dependent and molecular-dependent algorithms proposed by Van den Beld *et al.*, and this by configuring the cgMLST-based clustering of inconclusive isolates with reference strains [36].

3. Genoserotyping:

The aforementioned issues of phenotypic serotyping promote the development of several molecular techniques allowing the detection and characterization of isolates at the genetic level regardless if the genetic material was expressed or not. Monitoring the disease burden requires fast and high throughput methods allowing identification and surveillance at the serotype level. Although the WGS will complement or replace conventional serotyping of *Shigella* soon, it is not ready for routine use in most clinical microbiological laboratories. Generally, the molecular serotyping techniques are considered as fast methods generating a deluge of objective information in a relatively short period because of their high-throughput capabilities.

In brief, molecular serotyping was firstly applied to *Shigella* by Coimbra *et al.*, that suggested the restriction using the enzyme MboII of an amplified *rfb* region clustering most O-antigen encoding genes to decipher the serotype-specific *rfb* polymorphism [76]. This technique also called *rfb*-RFLP had shown a closer resolution to full traditional serotyping scheme generating discernable O-antigen patterns for each serotype except for *S. boydii* 12, which showed two distinct patterns, and *S. flexneri* serotypes 1–5, X and Y, which all gave the same indistinguishable pattern [76]. To ensure a quick identification at the serotype level, a dynamic software (Molecular serotyping tool) was then developed to compare the *rfb*-RFLP

patterns of clinical isolates to those in a database encompassing patterns of previously 171 known O-antigens of *Shigella* and *E. coli* [77]. Furthermore, copious multiplex PCR schemes have been established for *Shigella* serotyping as quick affordable methods, especially for *S. flexneri*. Sun *et al.,* developed a single tube multiplex PCR assay with eight sets of pair primers targeting O-antigen synthesis genes and modification genes, that allowed the identification of 14 out 15 serotypes of *S. flexneri* (except serotype Xv) with a high concordance (97.8%) with traditional slide agglutination method [78]. This conventional PCR was also upgraded into a real-time version [79]. Evaluation studies proved its full correlation with WGS and its outperformance over traditional methods wherein discrepancies between phenotypic and genotypic techniques were ascribed to the presence of novel genotypes, non-specific cross-reactions, or genetic modifications in O-antigen synthesis or modification genes [80]. Besides, two other designed multiplex PCR assays could efficiently determine the 19 serotypes of *S. flexneri* recognized so far where "PCR A" defined serotype genes and "PCR B" identified serotype 7 specific genes and group antigenic factors genes [81]. To outstrip the PCR multiplex-associated problems particularly the differentiation between similar-sized bands, Li *et al.,* developed a DNA microarray able to simultaneously detect 34 distinct O-antigen *Shigella* forms with high sensitivity and specificity rates [82]. However, these methods relying on O-antigen specific biosynthetic genes must be complemented with biochemical tests for a reliable differentiation, as many *Shigella* serotypes share identical O-antigen with commensal *E. coli*, as well as, the high level of observed recombination among serotype-specific genes mostly encoded on mobile genetic elements [37,83]. WGS provides new insights into the *Shigella* phylogeny never tackled before. By performing *in silico* molecular serotyping based on Sun *et al.,* 2011 scheme, Connor *et al.,* revealed that the serotype notion weakly predicted the phylogenetic relationships between strains of *S. flexneri* where each of the seven identified phylogenetical groups encompassed two or more serotypes [37,78]. Besides, WGS places the whole genetic repertoire under scrutiny and offers the ability to simultaneously interrogate many genes. Indeed, analyzing a sole genetic marker could mislead the identification at both species and serotypes level due to the considerable genomic variability of presumed specific genomic targets [34]. Additionally, WGS could maintain backward compatibility with historical data by providing a framework for *in silico* genome-derived serotyping, along with its ability to identify novel serotypes. In this line, after a profound examination of 259 *Shigella* genomes belonging to 53 serotypes; Wu *et al.,* recently developed an automated pipeline, ShigaTyper, able to quickly identify and predict 59 serotypes from Illumina paired-end reads with high accuracy (98.2%) [34]. Likewise, Ventola *et al.,* proposed two novel tools to be

implemented in the National Reference of *Salmonella* and *Shigella* of Belgium for *Shigella* surveillance: The first tool consisted of a cost-effective Luminex assay based on a modular multiplex oligonucleotide ligation-PCR procedure targeting five genetic markers for species identification and elven serotype markers for *S. sonnei* and *S. flexneri*, in a single test [70,84]. The second tool is a WGS-based workflow for automated prediction of *Shigella* serotypes with a particular hint on gene functionality [70].

Conclusion

Shigella identification and serotyping remain a thorny issue and a daunting task, especially in developing countries [62]. The application of low resolving techniques profoundly impacts the *Shigella* epidemiology as well as the accurate assessment of the appropriate treatment in patients with shigellosis. While DNA-based methods are increasingly becoming a common practice, they still suffer from low specificity and many methods should be combined to elucidate the real identity of an isolate. Our review supports the superior discriminatory power of WGS for both, the identification and serotyping of *Shigella* spp., as well as its excellence in disclosing novel genomic markers, and validating previously well-established methods on large diverse genomic collections. Incontrovertibly, WGS (will) become the future gold standard for the *Shigella* surveillance and epidemiologic investigations, particularly with the steady decreasing cost of sequencing platforms and the growing number of user-friendly bioinformatics tools and pipelines. Meanwhile, appropriate backward compatibility should be maintained to harmonize language between the different stakeholders and establish firm bridges with historical data.

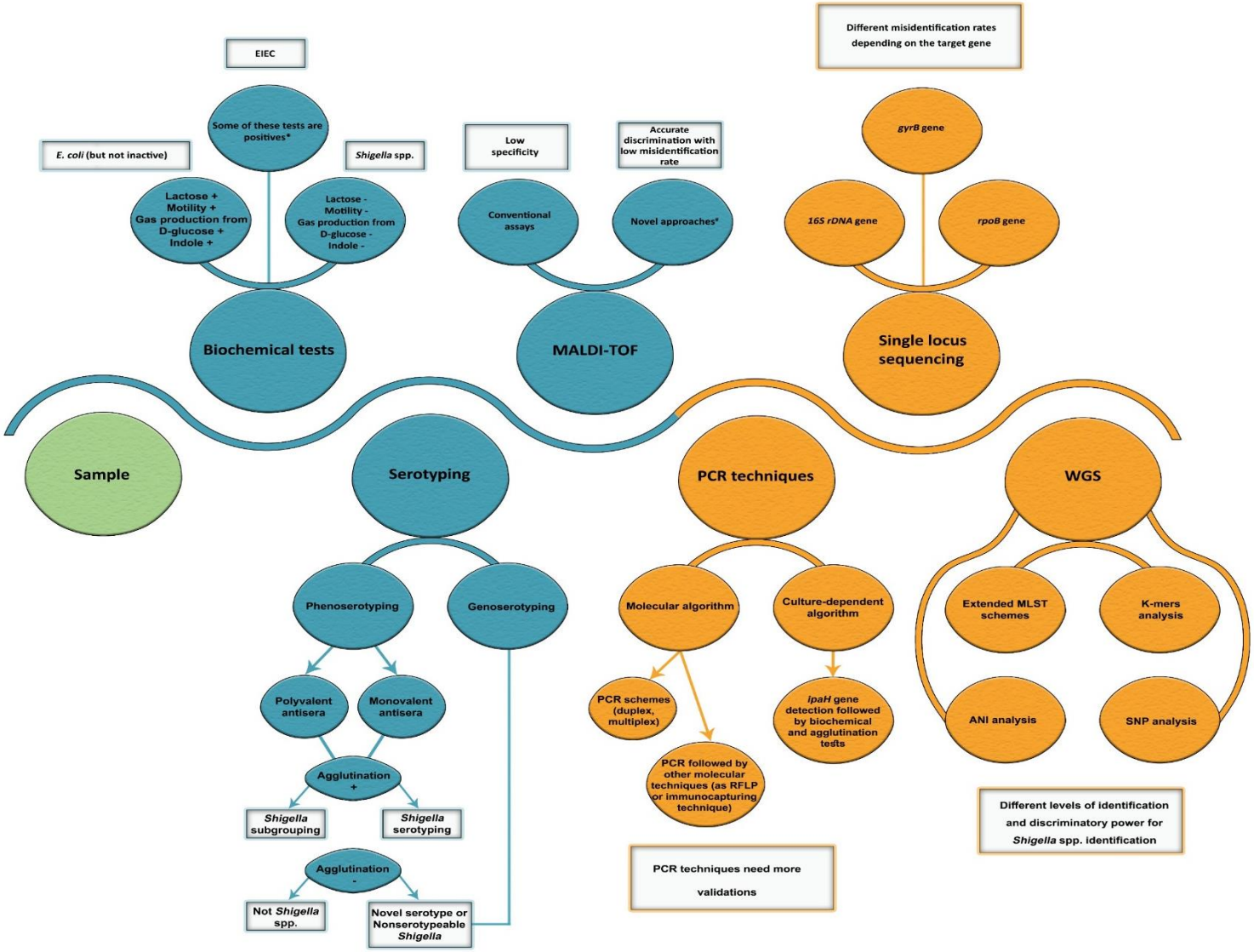

Figure 1: *Shigella* identification methods and strategies. Blue: Phenotypic Techniques, Yellow: Molecular Techniques. * : EIEC can be differentiated from *Shigella* only by a very limited number of tests, which include motility, mucate, and salicin fermentation, esculin hydrolysis, the combined positivity of indole production and gas formation form D-glucose, and acetate utilization.

Table 1: Summary of the most frequently used methods for *Shigella* identification.

Technique		Target	Ability to distinguish between *Shigella* and *E. coli*	References
Phenotypic	Biochemical tests	The enzymatic activity and the ability to ferment some sugars	Inability to distinguish between *Shigella* spp. and other genera, *Hafnia, Providencia*, atypical *E. coli* and non-lactose fermenting or anaerogenic *E. coli.*	[85]
	Serology	The O antigen present in the surface of bacteria	Occurrence of intra- and inter-species cross-reactions, presence of occasional provisional *Shigella* serovars, serotype conversion, untypable strains, not illustrative of phylogenetic relationships.	[83]
	MALDI-TOF MS	The spectra of ribosomal proteins signature	Inability of conventional MALDI-TOF MS assays to discriminate between *Shigella* and *E. coli.* Advanced software or customized reference library could distinguish inactive and other non-lactose-fermenting *E. coli from Shigella* spp.	[30,40,42]
Molecular	PCR techniques	*ipaH*, and *lacY*	Differentiation between *Shigella* (*ipaH*+, *lacY*-), EIEC (*ipaH*+, *lacY*+), noninvasive *E. coli* (*ipaH*-, *lacY*+), however, recent data demonstrate low performance.	[44,45,50]
		uidA, and *lacY*	Differentiation between *Shigella* (*uidA*+, *lacY*-), and *E. coli* including EIEC (*uidA*+, *lacY*+), however, recent data demonstrate low performance.	
	Single locus sequence-based	*16S rDNA* gene	Inability to distinguish *E. coli* from *Shigella* spp. due to the high sequence similarity (>99%)	[58]
		rpoB gene	Inability to distinguish *E. coli* from *Shigella* spp. due to the high sequence similarity (>99%)	[86]
		gyrB gene	Capability to differentiate between *E. coli* and *Shigella* spp., but further large validations are needed.	[57]
	WGS	K-mers	Accurately differentiate *E. coli* from *Shigella* to the species level, but some EIEC isolates can be misidentified as *S. flexneri* or *S. boydii.*	[64]
		SNP markers	Accurately differentiate EIEC from *Shigella* spp. with typing ability among strains of the same species.	[50]

		Average of nucleotide identity	Most of the ANI-based delineation tools are unable to delineate *Shigella* spp. from *E. coli*. Notably, the GGDC web tool with the ANI-f1 formula showed some power.	[65]
		Cg-MLST: conserved *loci* shared between *Shigella* and *E. coli* species	The cgMLST-based clustering differentiates between *E. coli* and *Shigella* spp. with high-resolution capabilities.	[75]

REFERENCES

1. Lampel KA, Formal SB, Maurelli AT. A Brief History of *Shigella*. EcoSal Plus 2018;8. doi: 10.1128/ecosalplus.ESP-0006-2017

2. Baker S, The HC. Recent insights into *Shigella*. Curr. Opin. Infect. Dis. 2018;31:449–54. doi: 10.1097/QCO.0000000000000475

3. McCrickard LS, Crim SM, Kim S, Bowen A. Disparities in severe shigellosis among adults - Foodborne diseases active surveillance network, 2002-2014. BMC Public Health 2018;18:221. doi: 10.1186/s12889-018-5115-4

4. Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB. Clinical trials of *Shigella* vaccines: two steps forward and one step back on a long, hard road. Nat. Rev. Microbiol. 2007;5:540–53. doi: 10.1038/nrmicro1662

5. Mattock E, Blocker AJ. How Do the Virulence Factors of *Shigella* Work Together to Cause Disease? Front. Cell. Infect. Microbiol. 2017;7. doi: 10.3389/fcimb.2017.00064

6. Kotloff KL, Riddle MS, Platts-Mills JA, Pavlinac P, Zaidi AKM. Shigellosis. Lancet Lond. Engl. 2018;391:801–12. doi: 10.1016/S0140-6736(17)33296-8

7. Sansonetti PJ. III. Shigellosis: from symptoms to molecular pathogenesis. Am. J. Physiol.-Gastrointest. Liver Physiol. 2001;280:G319–23. doi: 10.1152/ajpgi.2001.280.3.G319

8. Peng J, Yang J, Jin Q. The molecular evolutionary history of *Shigella* spp. and enteroinvasive *Escherichia coli*. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 2009;9:147–52. doi: 10.1016/j.meegid.2008.10.003

9. Dodd CE, Jones D. A numerical taxonomic study of the genus Shigella. J. Gen. Microbiol. 1982;128:1933–57. doi: 10.1099/00221287-128-9-1933

10. Kang C-H, Nam Y-D, Chung W-H, Quan Z-X, Park Y-H, Park S-J, et al. Relationship between genome similarity and DNA-DNA hybridization among closely related bacteria. J. Microbiol. Biotechnol. 2007;17:945–51.

11. Pupo GM, Lan R, Reeves PR. Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc. Natl. Acad. Sci. U. S. A. 2000 12;97:10567–72. doi: 10.1073/pnas.180094797

12. Lan R, Reeves PR. *Escherichia coli* in disguise: molecular origins of Shigella. Microbes Infect. 2002;4:1125–32.

13. Lukjancenko O, Wassenaar TM, Ussery DW. Comparison of 61 Sequenced *Escherichia coli* Genomes. Microb. Ecol. 2010;60:708. doi: 10.1007/s00248-010-9717-3

14. Reeves PR, Liu B, Zhou Z, Li D, Guo D, Ren Y, et al. Rates of mutation and host transmission for an *Escherichia coli* clone over 3 years. PloS One 2011;6:e26907. doi:

10.1371/journal.pone.0026907

15. Zuo G, Xu Z, Hao B. *Shigella* strains are not clones of *Escherichia coli* but sister species in the genus *Escherichia*. Genomics Proteomics Bioinformatics 2013;11:61–5. doi: 10.1016/j.gpb.2012.11.002

16. Hao B, Qi J. Prokaryote phylogeny without sequence alignment: from avoidance signature to composition distance. J. Bioinform. Comput. Biol. 2004;2:1–19. doi: 10.1142/s0219720004000442

17. Zuo G, Hao B. CVTree3 Web Server for Whole-genome-based and Alignment-free Prokaryotic Phylogeny and Taxonomy. Genomics Proteomics Bioinformatics 2015;13:321. doi: 10.1016/j.gpb.2015.08.004

18. Ud-Din A, Wahid S. Relationship among *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC) and their differentiation. Braz J Microbiol. 2015 4;45:1131–8. doi: 10.1590/s1517-83822014000400002

19. Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW. Manual of Clinical Microbiology, 10th Edition [Internet]. 10th ed. American Society of Microbiology; 2011 [cited 2020 4]. doi: https://www.asmscience.org/content/book/10.1128/9781555816728doi: 10.1128/9781555816728

20. The HC, Thanh DP, Holt KE, Thomson NR, Baker S. The genomic signatures of *Shigella* evolution, adaptation and geographical spread. Nat. Rev. Microbiol. 2016 29;14:235–50. doi: 10.1038/nrmicro.2016.10

21. Lan R, Alles MC, Donohoe K, Martinez MB, Reeves PR. Molecular Evolutionary Relationships of Enteroinvasive *Escherichia coli* and *Shigella* spp. Infect. Immun. 2004;72:5080–8. doi: 10.1128/IAI.72.9.5080-5088.2004

22. van den Beld MJC, Reubsaet F a. G. Differentiation between *Shigella*, enteroinvasive *Escherichia coli* (EIEC) and noninvasive *Escherichia coli*. Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol. 2012;31:899–904. doi: 10.1007/s10096-011-1395-7

23. van den Beld MJC, Warmelink E, Friedrich AW, Reubsaet FAG, Schipper M, de Boer RF, et al. Incidence, clinical implications and impact on public health of infections with *Shigella* spp. and entero-invasive *Escherichia coli* (EIEC): results of a multicenter cross-sectional study in the Netherlands during 2016-2017. BMC Infect. Dis. 2019 9;19:1037. doi: 10.1186/s12879-019-4659-y

24. Dekker JP, Frank KM. *Salmonella*, *Shigella*, and *Yersinia*. Clin. Lab. Med. 2015;35:225–46. doi: 10.1016/j.cll.2015.02.002

25. O'Hara CM. Manual and automated instrumentation for identification of *Enterobacteriaceae* and other aerobic gram-negative bacilli. Clin. Microbiol. Rev. 2005;18:147–62. doi: 10.1128/CMR.18.1.147-162.2005

26. Holmes B, Willcox WR, Lapage SP. Identification of *Enterobacteriaceae* by the API 20E system. J. Clin. Pathol. 1978;31:22–30. doi: 10.1136/jcp.31.1.22

27. Ekwall E, Dimander M. Comparison of micro-ID with API 20E for identification of *Salmonella*, *Shigella* and *Yersinia* species. Eur. J. Clin. Microbiol. 1982;1:134–7. doi: 10.1007/bf02019612

28. Carroll KC, Glanz BD, Borek AP, Burger C, Bhally HS, Henciak S, et al. Evaluation of the BD Phoenix automated microbiology system for identification and antimicrobial susceptibility testing of *Enterobacteriaceae*. J. Clin. Microbiol. 2006;44:3506–9. doi: 10.1128/JCM.00994-06

29. Snyder JW, Munier GK, Johnson CL. Direct comparison of the BD phoenix system with the MicroScan WalkAway system for identification and antimicrobial susceptibility testing of *Enterobacteriaceae* and nonfermentative gram-negative organisms. J. Clin. Microbiol. 2008;46:2327–33. doi: 10.1128/JCM.00075-08

30. Khot PD, Fisher MA. Novel approach for differentiating *Shigella* species and *Escherichia coli* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 2013;51:3711–6. doi: 10.1128/JCM.01526-13

31. Gupta S, Aruna C, Muralidharan S. Misidentification of a commensal inactive *Escherichia coli* as *Shigella* sonnei by an automated system in a critically ill patient. Clin. Lab. 2011;57:767–9.

32. O'Hara CM, Miller JM. Evaluation of the MicroScan rapid neg ID3 panel for identification of *Enterobacteriaceae* and some common gram-negative nonfermenters. J. Clin. Microbiol. 2000;38:3577–80.

33. van der Ploeg CA, Viñas MR, Terragno R, Bruno SB, Binsztein N. Laboratory Protocol: Serotyping of *Shigella* spp. Geneva [Internet]. 2010 26;doi: http://www.antimicrobialresistance.dk/data/images/protocols/gfn_shigellaserotypification-final-29-06-10.pdf

34. Wu Y, Lau HK, Lee T, Lau DK, Payne J. In Silico Serotyping Based on Whole-Genome Sequencing Improves the Accuracy of *Shigella* Identification. Appl. Environ. Microbiol. 2019;85. doi: 10.1128/AEM.00165-19

35. Muthuirulandi Sethuvel DP, Devanga Ragupathi NK, Anandan S, Veeraraghavan B. Update on: *Shigella* new serogroups/serotypes and their antimicrobial resistance. Lett. Appl.

Microbiol. 2017;64:8–18. doi: 10.1111/lam.12690

36. van den Beld MJC, de Boer RF, Reubsaet FAG, Rossen JWA, Zhou K, Kuiling S, et al. Evaluation of a Culture-Dependent Algorithm and a Molecular Algorithm for Identification of *Shigella* spp., *Escherichia coli*, and Enteroinvasive *E. coli*. J. Clin. Microbiol. 2018;56. doi: 10.1128/JCM.00510-18

37. Connor TR, Barker CR, Baker KS, Weill F-X, Talukder KA, Smith AM, et al. Species-wide whole genome sequencing reveals historical global spread and recent local persistence in *Shigella flexneri*. eLife 2015 4;4:e07335. doi: 10.7554/eLife.07335

38. Sun Q, Lan R, Wang J, Xia S, Wang Y, Wang Y, et al. Identification and Characterization of a Novel *Shigella flexneri* Serotype Yv in China. PLoS ONE [Internet] 2013 30 [cited 2020 5];8. doi: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3728103/doi: 10.1371/journal.pone.0070238

39. Allison GE, Verma NK. Serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri*. Trends Microbiol. 2000;8:17–23. doi: 10.1016/s0966-842x(99)01646-7

40. Rahi P, Prakash O, Shouche YS. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass-Spectrometry (MALDI-TOF MS) Based Microbial Identifications: Challenges and Scopes for Microbial Ecologists. Front. Microbiol. 2016;7:1359. doi: 10.3389/fmicb.2016.01359

41. Almuzara M, Barberis C, Traglia G, Famiglietti A, Ramirez MS, Vay C. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry for species identification of nonfermenting Gram-negative bacilli. J. Microbiol. Methods 2015;112:24–7. doi: 10.1016/j.mimet.2015.03.004

42. Paauw A, Jonker D, Roeselers G, Heng JME, Mars-Groenendijk RH, Trip H, et al. Rapid and reliable discrimination between *Shigella* species and *Escherichia coli* using MALDI-TOF mass spectrometry. Int. J. Med. Microbiol. IJMM 2015;305:446–52. doi: 10.1016/j.ijmm.2015.04.001

43. Ling J, Wang H, Li G, Feng Z, Song Y, Wang P, et al. A novel short-term high-lactose culture approach combined with a matrix-assisted laser desorption ionization-time of flight mass spectrometry assay for differentiating *Escherichia coli* and *Shigella* species using artificial neural networks. PloS One 2019;14:e0222636. doi: 10.1371/journal.pone.0222636

44. Pavlovic M, Luze A, Konrad R, Berger A, Sing A, Busch U, et al. Development of a duplex real-time PCR for differentiation between *E. coli* and *Shigella* spp. J. Appl. Microbiol. 2011;110:1245–51. doi: 10.1111/j.1365-2672.2011.04973.x

45. Løbersli I, Wester AL, Kristiansen Å, Brandal LT. Molecular Differentiation of *Shigella* Spp. from Enteroinvasive *E. coli*. Eur. J. Microbiol. Immunol. 2016 29;6:197–205. doi: 10.1556/1886.2016.00004

46. Theron J, Morar D, Du Preez M, Brözel VS, Venter SN. A sensitive seminested PCR method for the detection of *Shigella* in spiked environmental water samples. Water Res. 2001 1;35:869–74. doi: 10.1016/S0043-1354(00)00348-1

47. Jackson MP. Detection of Shiga toxin-producing *Shigella dysenteriae* type 1 and *Escherichia coli* by using polymerase chain reaction with incorporation of digoxigenin-11-dUTP. J. Clin. Microbiol. 1991 1;29:1910–4.

48. Kim H-J, Ryu J-O, Song J-Y, Kim H-Y. Multiplex Polymerase Chain Reaction for Identification of Shigellae and Four *Shigella* Species Using Novel Genetic Markers Screened by Comparative Genomics. Foodborne Pathog. Dis. 2017;14:400–6. doi: 10.1089/fpd.2016.2221

49. Ashida H, Sasakawa C. *Shigella* IpaH Family Effectors as a Versatile Model for Studying Pathogenic Bacteria. Front. Cell. Infect. Microbiol. 2015;5:100. doi: 10.3389/fcimb.2015.00100

50. Pettengill EA, Pettengill JB, Binet R. Phylogenetic Analyses of *Shigella* and Enteroinvasive *Escherichia coli* for the Identification of Molecular Epidemiological Markers: Whole-Genome Comparative Analysis Does Not Support Distinct Genera Designation. Front. Microbiol. 2015;6:1573. doi: 10.3389/fmicb.2015.01573

51. Devanga Ragupathi NK, Muthuirulandi Sethuvel DP, Inbanathan FY, Veeraraghavan B. Accurate differentiation of *Escherichia coli* and *Shigella* serogroups: challenges and strategies. New Microbes New Infect. 2018;21:58–62. doi: 10.1016/j.nmni.2017.09.003

52. Ojha SC, Yean Yean C, Ismail A, Banga Singh K-K. A Pentaplex PCR Assay for the Detection and Differentiation of *Shigella* Species [Internet]. BioMed Res. Int. 2013 [cited 2020 17];2013:e412370. doi: https://www.hindawi.com/journals/bmri/2013/412370/doi: https://doi.org/10.1155/2013/412370

53. Sahl JW, Morris CR, Emberger J, Fraser CM, Ochieng JB, Juma J, et al. Defining the phylogenomics of *Shigella* species: a pathway to diagnostics. J. Clin. Microbiol. 2015;53:951–60. doi: 10.1128/JCM.03527-14

54. Kingombe CIB, Cerqueira-Campos M-L, Farber JM. Molecular strategies for the detection, identification, and differentiation between enteroinvasive *Escherichia coli* and *Shigella* spp. J. Food Prot. 2005;68:239–45. doi: 10.4315/0362-028x-68.2.239

55. Peng X, Luo W, Zhang J, Wang S, Lin S. Rapid Detection of *Shigella* Species in

Environmental Sewage by an Immunocapture PCR with Universal Primers. Appl. Environ. Microbiol. 2002 1;68:2580–3. doi: 10.1128/AEM.68.5.2580-2583.2002

56. Janda JM, Abbott SL. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. J. Clin. Microbiol. 2007 1;45:2761. doi: 10.1128/JCM.01228-07

57. Dhiviya Prabaa MS, Naveen Kumar DR, Yesurajan IF, Anandan S, Kamini W, Balaji V. Identification of nonserotypeable *Shigella* spp. using genome sequencing: a step forward. Future Sci. OA 2017;3:FSO229. doi: 10.4155/fsoa-2017-0063

58. Chen L, Cai Y, Zhou G, Shi X, Su J, Chen G, et al. Rapid Sanger sequencing of the 16S rRNA gene for identification of some common pathogens. PloS One 2014;9:e88886. doi: 10.1371/journal.pone.0088886

59. Adékambi T, Shinnick TM, Raoult D, Drancourt M. Complete *rpoB* gene sequencing as a suitable supplement to DNA-DNA hybridization for bacterial species and genus delineation. Int. J. Syst. Evol. Microbiol. 2008;58:1807–14. doi: 10.1099/ijs.0.65440-0

60. Fukushima M, Kakinuma K, Kawaguchi R. Phylogenetic analysis of Salmonella, *Shigella*, and *Escherichia coli* strains on the basis of the *gyrB* gene sequence. J. Clin. Microbiol. 2002;40:2779–85. doi: 10.1128/jcm.40.8.2779-2785.2002

61. Nochi Z, Sahebekhtiari N, Kharaziha P, Chiani M, Motavaze K, Dabiri H, et al. Comparison of 16S rRNA, 23S rRNA andgyrB genes sequences in phylogenetic relationships of*Shigella* isolates from Iran. Ann. Microbiol. 2009 1;59:615–22. doi: 10.1007/BF03175154

62. Halimeh FB, Rafei R, Diene S, Mikhael M, Mallat H, Achkar M, et al. Challenges in identification of enteroinvasive *Escherichia coli* and *Shigella* spp. in Lebanon. Acta Microbiol. Immunol. Hung. 2020 30;1–7. doi: 10.1556/030.2020.01102

63. Wetterstrand KA. DNA Sequencing Costs: Data [Internet]. Genome.gov 2020 21 [cited 2020 21];doi: https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data

64. Chattaway MA, Schaefer U, Tewolde R, Dallman TJ, Jenkins C. Identification of *Escherichia coli* and *Shigella* Species from Whole-Genome Sequences. J. Clin. Microbiol. 2017;55:616–23. doi: 10.1128/JCM.01790-16

65. Maderankova D, Jugas R, Sedlar K, Vitek M, Skutkova H. Rapid Bacterial Species Delineation Based on Parameters Derived From Genome Numerical Representations. Comput. Struct. Biotechnol. J. 2019 9;17:118–26. doi: 10.1016/j.csbj.2018.12.006

66. Uelze L, Grützke J, Borowiak M, Hammerl JA, Juraschek K, Deneke C, et al. Typing methods based on whole genome sequencing data. One Health Outlook 2020 18;2:3. doi:

10.1186/s42522-020-0010-1

67. Rafei R, Osman M, Dabboussi F, Hamze M. Update on the epidemiological typing methods for *Acinetobacter baumannii*. Future Microbiol. 2019;14:1065–80. doi: 10.2217/fmb-2019-0134

68. Chattaway MA, Greig DR, Gentle A, Hartman HB, Dallman TJ, Jenkins C. Whole-Genome Sequencing for National Surveillance of *Shigella flexneri*. Front. Microbiol. [Internet] 2017 [cited 2020 28];8. doi: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01700/fulldoi: 10.3389/fmicb.2017.01700

69. Hayford AE, Mammel MK, Lacher DW, Brown EW. Single nucleotide polymorphism (SNP)-based differentiation of *Shigella* isolates by pyrosequencing. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 2011;11:1761–8. doi: 10.1016/j.meegid.2011.07.015

70. Ventola E, Bogaerts B, De Keersmaecker SCJ, Vanneste K, Roosens NHC, Mattheus W, et al. Shifting national surveillance of *Shigella* infections toward geno-serotyping by the development of a tailored Luminex assay and NGS workflow. MicrobiologyOpen 2019;8:e00807. doi: 10.1002/mbo3.807

71. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. 2006;60:1136–51. doi: 10.1111/j.1365-2958.2006.05172.x

72. Weihong Qi, Lacher DW, Bumbaugh AC, Hyma KE, Ouellette LM, Large TM, et al. EcMLST: an online database for multi locus sequence typing of pathogenic *Escherichia coli* [Internet]. In: Proceedings. 2004 IEEE Computational Systems Bioinformatics Conference, 2004. CSB 2004. Stanford, CA, USA: IEEE; 2004 [cited 2020 5]. p. 499–500.doi: http://ieeexplore.ieee.org/document/1332482/doi: 10.1109/CSB.2004.1332482

73. Jaureguy F, Landraud L, Passet V, Diancourt L, Frapy E, Guigon G, et al. Phylogenetic and genomic diversity of human bacteremic *Escherichia coli* strains. BMC Genomics 2008 26;9:560. doi: 10.1186/1471-2164-9-560

74. Maiden MCJ, Jansen van Rensburg MJ, Bray JE, Earle SG, Ford SA, Jolley KA, et al. MLST revisited: the gene-by-gene approach to bacterial genomics. Nat. Rev. Microbiol. 2013;11:728–36. doi: 10.1038/nrmicro3093

75. Kimura B. Will the emergence of core genome MLST end the role of in silico MLST? Food Microbiol. 2018;75:28–36. doi: 10.1016/j.fm.2017.09.003

76. Coimbra RS, Grimont F, Grimont PA. Identification of *Shigella* serotypes by restriction of amplified O-antigen gene cluster. Res. Microbiol. 1999;150:543–53. doi: 10.1016/s0923-

2508(99)00103-5

77. Coimbra RS, Artiguenave F, Jacques LSRZ, Oliveira GC. MST (molecular serotyping tool): a program for computer-assisted molecular identification of *Escherichia coli* and *Shigella* O antigens. J. Clin. Microbiol. 2010;48:1921–3. doi: 10.1128/JCM.00357-10

78. Sun Q, Lan R, Wang Y, Zhao A, Zhang S, Wang J, et al. Development of a Multiplex PCR Assay Targeting O-Antigen Modification Genes for Molecular Serotyping of *Shigella flexneri* ▿. J. Clin. Microbiol. 2011;49:3766–70. doi: 10.1128/JCM.01259-11

79. Gentle A, Ashton PM, Dallman TJ, Jenkins C. Evaluation of Molecular Methods for Serotyping *Shigella flexneri*. J. Clin. Microbiol. 2016;54:1456. doi: 10.1128/JCM.03386-15

80. Brengi SP, Sun Q, Bolaños H, Duarte F, Jenkins C, Pichel M, et al. PCR-Based Method for *Shigella flexneri* Serotyping: International Multicenter Validation. J. Clin. Microbiol. 2019;57. doi: 10.1128/JCM.01592-18

81. van der Ploeg CA, Rogé AD, Bordagorría XL, de Urquiza MT, Castillo ABC, Bruno SB. Design of Two Multiplex PCR Assays for Serotyping *Shigella flexneri*. Foodborne Pathog. Dis. 2018;15:33–8. doi: 10.1089/fpd.2017.2328

82. Li Y, Cao B, Liu B, Liu D, Gao Q, Peng X, et al. Molecular detection of all 34 distinct O-antigen forms of *Shigella*. J. Med. Microbiol. 2009;58:69–81. doi: 10.1099/jmm.0.000794-0

83. Liu B, Knirel YA, Feng L, Perepelov AV, Senchenkova SN, Wang Q, et al. Structure and genetics of *Shigella* O antigens. FEMS Microbiol. Rev. 2008;32:627–53. doi: 10.1111/j.1574-6976.2008.00114.x

84. Wuyts V, Roosens NHC, Bertrand S, Marchal K, De Keersmaecker SCJ. Guidelines for Optimisation of a Multiplex Oligonucleotide Ligation-PCR for Characterisation of Microbial Pathogens in a Microsphere Suspension Array [Internet]. BioMed Res. Int. 2015 [cited 2020 12];2015:e790170. doi: https://www.hindawi.com/journals/bmri/2015/790170/doi: https://doi.org/10.1155/2015/790170

85. Hawkey PM. Identification of *Enterobacteriaceae* [Internet]. In: Principles and Practice of Clinical Bacteriology. John Wiley & Sons, Ltd; 2006 [cited 2019 28]. p. 341–5.doi: https://onlinelibrary.wiley.com/doi/abs/10.1002/9780470017968.ch27doi: 10.1002/9780470017968.ch27

86. Vos M, Quince C, Pijl AS, de Hollander M, Kowalchuk GA. A comparison of *rpoB* and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. PloS One 2012;7:e30600. doi: 10.1371/journal.pone.0030600

Article

Challenges in identification of enteroinvasive *Escherichia coli* and *Shigella* spp. in Lebanon

Fatima Bachir Halimeh[1,2], Rayane Rafei[1], Seydina Diene[2], Mary Mikhael[1], Hassan Mallat[1], Marcel Achkar[3], Fouad Dabboussi[1], Monzer Hamze[1*], Jean-Marc Rolain[2]

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[1] Laboratoire Microbiologie Santé et Environnement (LMSE), Doctoral School of Science and Technology, Faculty of Public Health, Lebanese University, Tripoli, Lebanon

[2] Aix Marseille University, IRD, APHM, MEPHI, IHU Méditerranée Infection, Faculté de Médecine et de Pharmacie, 19-21 Boulevard Jean Moulin, 13385 Marseille CEDEX 05, France

[3] Laboratory of Clinical Biology, NINI Hospital, Tripoli, Lebanon

* Corresponding author

AKADÉMIAI KIADÓ

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ORIGINAL ARTICLE

Escherichia coli and *Shigella* spp. in Lebanon

FATIMA BACHIR HALIMEH[1,2], RAYANE RAFEI[1], SEYDINA DIENE[2], MARY MIKHAEL[1], HASSAN MALLAT[1], MARCEL ACHKAR[3], FOUAD DABBOUSSI[1], MONZER HAMZE[1p] and JEAN-MARC ROLAIN[2]

[1] Laboratoire Microbiologie Santé et Environnement (LMSE), Doctoral School of Science and Technology, Faculty of Public Health, Lebanese University, Tripoli, Lebanon

[2] Aix Marseille University, IRD, APHM, MEPHI, IHU Méditerranée Infection, Faculté de Médecine et de Pharmacie, 19-21 Boulevard Jean Moulin, 13385 Marseille CEDEX 05, France

[3] Laboratory of Clinical Biology, NINI Hospital, Tripoli, Lebanon

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ABSTRACT

This study aimed to evaluate the routine identification tools available in Lebanon for differentiation of *Escherichia coli* and *Shigella* spp. The identification of 43 isolates defined as *Shigella* spp. by Api 20E was accessed using MALDI-TOF, serological testing, duplex PCR targeting *ipaH* (present in *Shigella* spp. and enteroinvasive *E. coli* "EIEC") and *lacY* (found in *E. coli* including EIEC but not *Shigella* spp.) as well as *gyrB* gene sequencing. Antibiotic susceptibility was investigated as well as Shiga-toxin production. All isolates were identified as *E. coli* by MALDI-TOF while the PCR showed a disparate group of 26 EIEC, 11 *Shigella* spp., 5 *E. coli* and 1 inactive *E. coli*. However, the sequencing of *gyrB* gene, which was recently described as a suitable marker for distinguishing *E. coli* and *Shigella* spp., identified all isolates as *E. coli*. Antibiotic resistance was noticeable against ß-lactams, rifampicin, trimethoprim-sulfamethoxazole, gentamicin, and ciprofloxacin. The most common variants of beta-lactamase genes were $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-3}}$. A great discordance between the used methods in identification was revealed herein. An accurate identification technique able to distinguish *E. coli* from *Shigella* spp. in routine laboratories is a pressing need in order to select the appropriate treatment and assess the epidemiology of these bacteria.

KEYWORDS

Shigella spp., *E*scherichia *coli*, EIEC, identification, Lebanon, phenotypic techniques, molecular techniques

INTRODUCTION

Diarrheal diseases constitute a major public health issue worldwide. Children under the age of five years represent the most clinical cases, moreover gastroenteritis is ranked as the second leading cause of mortality among them, accounting for approximately 526,000 death toll in 2015 [1, 2]. Microbial and host characteristics such as the inoculum size, acidity resistance and host immunity are among factors that enhance the transmission of enteric diseases and consequently their widespread occurrence [3, 4].

Diarrheal diseases are caused by a variety of etiological agents (viral, parasitic, and bacterial). Among bacterial etiological agents, *Escherichia coli* and *Shigella* spp. are two of the most common cause of bacterial diarrheal diseases [5]. The genetic closeness between these organisms led many researchers to consider them as *E. coli* clones, albeit the distinctive morphological, biochemical, and serological features [6]. Meanwhile, *Shigella* spp. and EIEC (enteroinvasive *E. coli*) share similar genetic (analogous virulence plasmid) and biochemical features [7]. Unlike *E. coli*, *Shigella* isolates are less active biochemically and react with limited

*Corresponding author.
E-mail: mhamze@monzerhamze.com

Table 1. The *gyrB* primers used for the standard PCR and sequencing

Primers	Nucleotide sequence	Annealing temperature (8C)
EC-SH-gyrB-F1	5'-TCACGCCGATAACTCTGTCT-3'	58.54
EC-SH-gyrB-R1	5'-ACTCTTTCACCAGCCAGTCC-3'	59.6
EC-SH-gyrB-F2	5'-TGGCTTCCAGGAAAACATCT-3'	57.02
EC-SH-gyrB-R2	5'-ATTTTCTGCGTCGCGTTGTA-3'	58.86
EC-SH-gyrB-F3	5'- GTGAAATGACCCGCCGTAAA-3'	58.84
EC-SH-gyrB-R3	5'- TCAACAGCAGCGTACGAATG-3'	58.93

set of antisera and shared with EIEC pathogenicity genes [8]. The high degree of relatedness was also validated by many techniques as multilocus sequence typing (MLST) and virulence genes sequencing [9]. In this context, the nomenclature of *Shigella* genus and its corresponding species has been kept for historical and medical reasons. Recently, the whole-genome-based, alignment-free and parameter-free CVTree approach showed that four established *Shigella* species form sister species to *E. coli* in the genus *Escherichia* [6]. This aforementioned contentious closeness between *E. coli* and *Shigella* spp. led to many challenges in their identification and differentiation in routine laboratories. Nowadays, many methods have been suggested to solve this dilemma as Duplex Real-Time Polymerase Chain Reaction (RT-PCR) targeting (*uidA* and *lacY* or *ipaH* and *lacY*), (MALDI-TOF MS) using an analysis software (ClinPro Tools Bruker Daltonics) [10–12] and even the Whole Genome Sequencing (WGS) followed by bioinformatics tools such as k-mers or Single Nucleotide Polymorphism [13, 14].

Similar to other developing countries, in Lebanon, the identification of Shigellosis is based on clinical manifestations and biochemical tests mainly the Api 20E gallery. Serological testing is performed only under request in specialized labs as LMSE (Laboratoire Microbiologie, Santé, et Environnement).

In our laboratory, we have a collection of 43 clinical isolates identified as *Shigella* spp. using Api 20E. After performing the serological and molecular analysis (PCR), our isolates are defined as a diverse group belonging to *Shigella* spp., EIEC, *E. coli*, and inactive *E. coli*. The aim of our study was to evaluate the used techniques in Lebanon for the identification of *Shigella* spp. and this by combining several molecular and phenotypic methods.

MATERIALS AND METHODS

Sample collection

A total of 43 isolates identified as *Shigella* spp. by API 20E gallery (BioMérieux, Marcy l'étoile, France) were recovered from clinical stool samples from North Lebanon between July, 2010 and September, 2016 (NINI Hospital). These isolates were conserved at the Laboratoire Microbiologie Santé et Environnement (LMSE).

Phenotypic, serological and molecular identification

Besides API 20E performed in the LMSE laboratory, identification was also ensured by MALDI-TOF MS (Bruker, Massachusetts, United States). Moreover, agglutination tests were made using Bio-Rad Antiserum antibodies (BIO-RAD, Marnes-la-Coquette, France). Molecular identification was performed by detecting two genes; *ipaH* (invasion plasmid antigen H coding gene) present in *Shigella* spp. and EIEC pathovar; and *lacY* (a lactose permease coding gene) present in the fermentative bacteria as *E. coli* [12]. The reference strains *E. coli* CSURP1570 [15] and *Shigella flexneri* DSMZ (DSM 4782-0317-001) were used as controls. Taking into account the similar species resolution of *gyrB* sequencing and the WGS [16], we designed two couples of primers for a conventional PCR and sequencing of *gyrB* for the identification of our isolates (Table 1). The DNA was extracted using the BioRobot EZ1 Advanced XL instrument (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A standard conventional PCR was carried out using EC-SH-gyrB-F1 and EC-SH-gyrB-R1 as external primers and the master mix QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany). The amplified fragment of 2kb total size is a conserved region between *E. coli* and *Shigella* isolates. Positive PCR products were purified by NucleoFast 96 PCR plate (Machery–Nagel EURL, France) and sequenced by BigDye terminator (California, United States) and EC-SH-gyrB-F2, EC-SH-gyrB-R2, EC-SH-gyrB-F3, and EC-SH-gyrB-R3 as internal specific primers (Table 1).

Shiga toxin molecular and enzyme immunoassay detection

Enzyme immunoassay SHIGA TOXIN QUIK CHEK (Alere™, TECHLAB®, Blacksburg, United States) was used to detect the STX using specific STX1 and STX2 antibodies according to the manufacturer's instructions. Real Time-PCR detecting *stx1* and *stx2* genes were carried out [17].

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was determined using the disk diffusion method on Mueller–Hinton agar, and the results were interpreted according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) [18]. The antibiotics tested were: Amoxicillin (AMX), Amoxicillin-clavulanic acid (AMC), Ticarcillin-

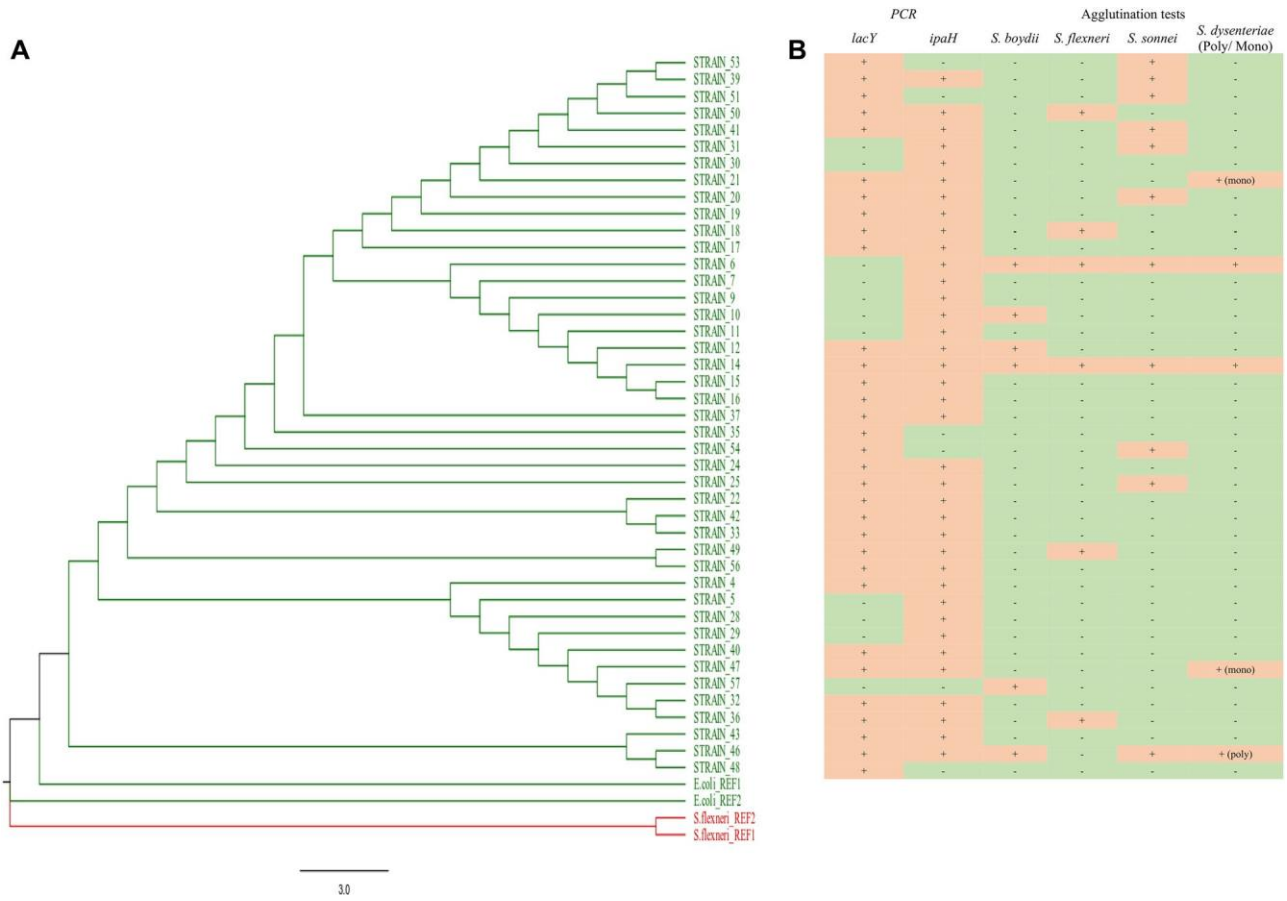

Figure 1. A: The Maximum Likehood phylogenetic tree of GyrB peptide sequences for the 43 isolates using MEGA 7 software with standard settings and 100 Bootstraps. The control strains (*S. flexneri* REF1) *Shigella flexneri* DSMZ (DSM 4782-0317-001) and (*E. coli* REF1) E. coli CSURP1570 he 2 NCBI reference strains (*S. flexneri* REF2) *Shigella flexneri* 2a_str_301_NC_0042272 and (*E. coli* REF2) *Escherichia coli*_str_k-12_subtr_MG1655_NC_0009133 were also analyzed. FigTree V1.4 was used to modify color of branches. B: The table represents the PCR detecting lacY and ipaH genes, faced to agglutination using antisera to determine the species of isolates

clavulanic acid (TCC), Cefotaxime (CTX), Cefoxitin (FOX), Aztreonam (ATM), Ertapenem (ETP), Imipenem (IMP), Trimethoprim-Sulfamethoxazole (SXT), Rifampicin (RIF), Ciprofloxacin (CIP), Gentamicin (GN), Fosfomycin (FF), Amikacin (AK), and Colistin (CT). The phenotypic confirmation of ESBL (Extended-spectrum b-lactamase) production was performed by the double-disk synergy test (DDST).

Detection of b-lactam resistance genes

The presence of b-lactamase genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) was detected by PCR using specific primers for: $bla_{CTX-M-1}$ group [19], $bla_{CTX-M-9}$ group [20], bla_{TEM} [21], bla_{SHV} [22] followed by sequencing.

RESULTS

Bacterial identification

All isolates were identified as *Shigella* spp. using the Gallery API 20E. The agglutination tests confirmed the *Shigella* identity for 17 isolates where 8, 3, 4, and 2 isolates reacted with *Shigella sonnei*, *Shigella boydii*, *S. flexneri*, and *Shigella dysenteriae* antisera respectively. Additionally, 2 isolates agglutinated with all species antisera, 1 isolate cross-reacted with *S. boydii*, *S. sonnei*, and *S. dysenteriae* antisera, and 23 isolates didn't react with any antisera. However, all were identified as *E. coli* by MALDI-TOF with a high score (2.21–2.46). On the other hand, the duplex PCR detecting *lacY* and *ipaH* revealed a heterogeneous collection of different species composed as follows: 26 isolates considered as EIEC (*lacY* positive, *ipaH* positive), 11 isolates as *Shigella* spp. (*lacY* negative, *ipaH* positive), 5 isolates as *E. coli* (*lacY* positive, *ipaH* negative) and one isolate as inactive *E. coli* (*lacY* negative, *ipaH* negative). This guides us to sequence the *gyrB* gene using our own designed primers. The *gyrB* gene sequencing demonstrated that all isolates were *E. coli*. The *S. flexneri* and *E. coli* control isolates were clearly distinguished. The *gyrB* gene similarity ranged from 96% to 98% between *S. flexneri* control isolates and the 43 isolates, while it was 99% between *E. coli* control isolate and our isolates. Moreover, the phylogenetic tree (Fig. 1A) shows a big cluster encompassing the 43 isolates with the *E. coli* control isolate and a NCBI reference *E. coli* (*Escherichia_coli*_str._K-12_substr._MG1655_NC_000913.3), while the *S. flexneri positive* control isolate is clustered with another *Shigella* isolate

Table 2. Antimicrobial resistance profiles and the b-lactamase encoding genes of studied isolates

Number of isolates	Resistance Profile	b-lactamase encoding genes		
		$bla_{CTX-M-15}$	$bla_{CTX-M-3}$	bla_{TEM-1}
2 isolates	AZT, AMC, CTX, TIM, RIF, GN	þ	–	þ
2 isolates	AMX, AZT, AMC, CTX, TIM, SXT, RIF, GN	þ	–	þ
2 isolates	AMX, AZT, AMC, CTX, TIM, SXT, RIF	–	þ	–
1 isolate	AMC, CTX, TIM, SXT, CIP, RIF	–	þ	–
1 isolate	AMC, SXT, RIF	–	–	þ
1 isolate	AZT, AMC, CTX, TIM, SXT, RIF, GN	þ	–	þ
1 isolate	AMX, AMC, TIM, RIF	–	–	þ
1 isolate	AMX, AMC, FOX, TIM, SXT, CIP, RIF	–	–	þ
1 isolate	AMX, AZT, AMC, CTX, FOX, TIM, SXT, RIF, GN	þ	–	þ
1 isolate	AMX, AZT, AMC, CTX, FOX, SXT, CIP, RIF	þ	–	–

from NCBI database (*Shigella_flexneri_*2a_str._301_ NC_004337.2).

Shiga toxin detection

The immunochromatoghraphy test revealed that none of the isolates was harbored neither STX1 nor STX2, and this result is confirmed by the real time-PCR showing a negative result for both genes (*stx1* and *stx2*).

Antibiotic susceptibility results

Most of the isolates showed reduced susceptibility to b-lactam antibiotics where 100%, 37.2%, 34.5%, 30.2%, 20.9%, and 18.6% were resistant to amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, amoxicillin, cefotaxime, cefoxitin, and aztreonam, respectively. In addition, rifampicin resistance was detected in 86% of isolates, trimethoprim-sulfamethoxazole resistance in 79% of isolates, gentamicin resistance in 14% of isolates, and ciprofloxacin in 7% of isolates. None of these isolates was resistant to carbapenem, amikacin, fosfomycin or colistin.

b-lactam resistance mechanisms

The double-disk synergy test (DDST) detected an ESBL production in 44.1% (19/43) of isolates showing a synergy translated by the shape "Bouchon de Champagne." Of 19 isolates phenotypically tested positive for ESBL, 16 were resistant to more than 3 antibiotics and 13 (68.4%) were ESBL positive by PCR. We detected the presence of the $bla_{CTX-M-1}$ group in 10 isolates (76.9%), and the bla_{TEM} group in 8 isolates (61.5%). None of the isolates had bla_{SHV} or $bla_{CTX-M-9}$ groups. Approximately, 46% of isolates carried 2 *bla* genes, while 54% of them harbored a single *bla* gene. The sequencing showed that 70% of the $bla_{CTX-M-1}$ group positive isolates were $bla_{CTX-M-15}$ and 30% of them were $bla_{CTX-M-3}$, while all the bla_{TEM} group positive isolates were bla_{TEM-1} (Table 2).

DISCUSSION

The close genetic relationship between *Shigella* spp. and *E. coli* is a scientific dilemma stumbling their accurate identification in the routine microbiological laboratories. Traditional biochemical and serological tests are the principal techniques used to identify these species in developing countries as Lebanon. However, many isolates as "inactive *E. coli* variants" cannot be identified using traditional or even molecular techniques such as conventional MALDI-TOF MS and 16S rRNA gene sequencing. Furthermore, *Shigella* isolates share their pathogenic genes with EIEC pathovar, thus complicating their clinical and laboratory diagnosis [23]. In this study, we aimed to evaluate the identification of 43 isolates identified as *Shigella* spp. using Api 20E and this by assessing a combination of phenotypic and molecular techniques. It's noteworthy to mention that 42 out of 43 isolates had LDC (lysine Desoxycarboxylase) negative test with Api 20E, a character considered negative in the *Shigella* genus. First of all, MALDI-TOF MS identified the isolates as *E. coli* with a high score (2.21–2.46). Indeed, the two phenotypic methods, namely Api 20E and MALDI-TOF MS, used different targets in order to unveil the identity of a particular bacterium. Gallery Api 20E's identification is based on the detection of the enzymatic activity and the fermentation of carbohydrates, while MALDI-TOF MS identifies bacteria through analysis of their proteins (mainly ribosomal proteins) in the mass range between 2,000 and 20,000 Daltons. Regarding their accuracy in identification, Api 20E has been qualified and preferred in many laboratories for the differentiation of *Enterobacteriaceae* family with a rate of correct identification reaching up to 97% [24, 25]. However, MALDI-TOF has a low-resolution power to distinguish between some taxonomic groups like *E. coli* and *Shigella* spp. [11, 26, 27]. Otherwise, a novel approaches approved by Bruker Daltonics (ClinPro Tools software), not used herein, can increase the taxonomic group resolution unachievable by methods like *16S rDNA* sequencing and routine MALDI-TOF MS [23, 28].

Due to this observed inconsistency, we have performed serological testing with traditional *Shigella* spp. antisera for these isolates. Serology, based on the detection of O antigen present in the outer membrane of Gram-negative bacteria linked to Lipopolysaccharide (LPS) backbone, has been considered till now as the reference technique for the identification of *Shigella* at species and serotype level. However, there were many examples of *Shigella* serotypes being misidentified through literature [29]. For example, *S. flexneri* serotype 6 was misidentified through history and was related phylogenetically to *S. boydii*. In addition, many O antigens of known *Shigella* serotypes are shared with some *E. coli* pathovars [13]. Indeed, *S. boydii* and *S. dysenteriae* share the same O antigen structure as that of EIEC leading thus to false positive results [13]. A recent paper unveiled the genetic causes behind the observed discordance between the traditional approach (serological testing combined with biochemical tests) and the k-mer identification derived from WGS. *S. flexneri* was misidentified by the traditional approach as *S. boydii* due to a dysfunctional $WZX_{1\text{-}5}$ gene [13]. Interestingly, a great percentage of our isolates were not typeable accounting for 53% of the total, a percentage higher those reported elsewhere in the world [30, 31].

Many of molecular techniques have been proposed to discriminate *Shigella* spp. from *E. coli*. The duplex PCR amplified the *lacY* gene (lactose permease gene) and either *uidA* (beta-glucuronidase encoding gene) or *ipaH* (invasion plasmid antigen H encoding gene) [10, 12]. The first schema targets the *uidA* found in both species and *lacY* particularly found in lactose-fermenting species like *E. coli*. Otherwise, the second schema can differentiate between *E. coli*, EIEC, and *Shigella* spp. The *lacY* gene is common in both *E. coli* and EIEC, while the *ipaH* gene is found in EIEC and *Shigella* spp. Also, *Shigella* spp. lacks the *lacY* gene [12]. In the present study, the performed duplex PCR revealed a diverse population among our isolates composed of *E. coli*, EIEC, inactive *E. coli*, and *Shigella* spp. Otherwise, this method can be also criticized. First, although some *Shigella* spp. lack the *lacY* gene, *S. dysenteriae* has *lacA* and *lacY* genes and *S. sonnei* has the three genes but they are unable to ferment lactose due to the lack of the permease activity [32]. Second, *ipaH* is considered as a virulence factor gene exclusively located on the virulence plasmid harbored by *Shigella* isolates. But the existence of several putative *ipaH* cognate genes in the chromosome is already mentioned [33, 34]. In this line, the presence of *ipaH* on the plasmid could lead to false positive or negative PCR results, due to the horizontal transfer of plasmid among the *Enterobacteriaceae* genera [28].

Comparing molecular results with the serological test, a huge discordance was noted. For example, among 26 isolates defined as EIEC by PCR, 12 reacted with *Shigella* spp. antisera. Additionally, of 5 isolates considered as *E. coli* by PCR, 3 reacted with *Shigella* spp. antisera. On the other hand, within 11 isolates identified as *Shigella* spp. by PCR, 7 didn't have any reaction with antisera (Fig. 1B). This discordance can be explained by the presence of common O antigen between *Shigella*, EIEC, and *E. coli* species [13]. Notoriously, a novel *Shigella* pathotype ST270; even considered as *Shigella* by k-mers derived by WGS, was identified as EIEC by traditional biochemical and serological test [35].

In the present study, the *gyr*B was able to identify correctly the *E. coli* and *Shigella* control strains, but it identified all the studied isolates as *E. coli*, even with the serological and biochemical evidence. Recently, gyrB gene, which encodes the subunit b protein of DNA gyrase (Topoisomerase type II protein), was considered as a suitable phylogenetic marker commonly used in the identification and classification of the evolutionary relationships of closely related species [36, 37]. Furthermore, a recent study demonstrated the ability of *gyr*B sequence in distinguishing the different *Shigella* isolates with percentages of divergence higher than found in 16S rRNA and 23S rRNA [38]. In *Shigella*, only one study described the comparable ability between *gyrB* gene sequencing and k-mer derived from WGS to distinguish *Shigella* from *E. coli* to the species level [16]. Other studies should be conducted to assess the use- fulness of this gene for the differentiation of *E. coli* and *Shigella* spp.

Moreover, in our study, we didn't detect any Shiga-toxin-producing isolates. Indeed, Shiga-toxin is commonly found in *S. dysenteriae* serotype 1 (SD1) and Shiga-Toxin *E. coli*. However, we had one isolate identified as *S. dysenteria*e by antisera agglutination and PCR, but it didn't produce the Shiga-toxin. This raises also substantial differences between the identification techniques.

Overall, the prevalence of ESBL-producing isolates was 30% (13/43), where $bla_{\text{TEM-1}}$ (69.2%) was the most common variants followed by $bla_{\text{CTX-M-15}}$ (53.8%), and $bla_{\text{CTX-M-3}}$ (23%). In Lebanon, *E. coli* constitutes about 54.7% of Gram-negative bacteria isolated from hospitalized patients, of which 32.1% harbored ESBL resistance genes [39]. In congruence with the worldwide situation, the rate of ESBL-producing *E. coli* isolates has shown an upward trend in Lebanon from 2% in 2003 to up to 33.6% in 2013, with ongoing increasing values [39, 40]. Compared to ESBL-producing *E. coli*, ESBL-producing *Shigella* isolates are less common worldwide, nevertheless, the rates vary according to countries and fluctuate between 1.5% and 68% [41–43].

CONCLUSION

In conclusion, although our study doesn't succeed to unravel the identity of isolates, we addressed here a real problem hindering the routine identification of *Shigella* spp. and *E. coli* in clinical microbiology laboratories, especially in low-income countries. Moreover, such misidentification can affect the accurate assessment of the appropriate treatment and the epidemiology of these bacteria. Indeed, if *Shigella* was misidentified as an *E. coli* from an extra-intestinal site, the treatment would be appropriate for an *E. coli* isolate. However, the susceptibility testing differs between these two organisms according to the CLSI (Clinical and Laboratory Standards Institute) guide where the first and second generation cephalosporins, cephamycin, and aminoglycosides

are not tested for *Shigella* isolates, as false *in vitro* susceptibility may occur [44]. Each used method herein seems to answer differently the tackling question, and an accurate method as WGS is highly needed to conclude about the identity of isolates and select the most appropriate method for species differentiation in low-income countries labs. One of the most helpful and rapid techniques that can be used to distinguish *E. coli* from *Shigella* spp. is Filmarray™ GI panel (BioMérieux, Marcy l'étoile, France) detecting 22 pathogens in less than 1 h with high sensitivity and specificity comparable to traditional laboratory methods [45].

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REFERENCES

[1] WHO. By category j Number of deaths by region – diarrhoeal diseases, http://apps.who.int/gho/data/view.main.CM1002015REG6-CH3?lang5en.

[2] Schroeder GN, Hilbi H. Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. Clin Microbiol Rev 2008; 21: 134–56.

[3] Lima IFN, Havt A, Lima AAM. Update on molecular epidemiology of *Shigella* infection. Curr Opin Gastroenterol 2015; 31: 30–7.

[4] Keusch GT, Walker CF, Das JK, Horton S, Habte D. Diarrheal diseases. In: Black RE, Laxminarayan R, Temmerman M, Walker N, editors. Reproductive, maternal, newborn, and child health: disease control priorities. 3rd ed., vol. 2. Washington (DC): The International Bank for Reconstruction and Development j The World Bank; 2016.

[5] Kotloff KL, Platts-Mills JA, Nasrin D, Roose A, Blackwelder WC, Levine MM. Global burden of diarrheal diseases among children in developing countries: incidence, etiology, and insights from new molecular diagnostic techniques. Vaccine 2017; 35: 6783–9.

[6] Zuo G, Xu Z, Hao B. *Shigella* strains are not clones of *Escherichia coli* but sister species in the genus *Escherichia*. Dev Reprod Biol 2013; 11: 61–5.

[7] The HC, Thanh DP, Holt KE, Thomson NR, Baker S. The genomic signatures of *Shigella* evolution, adaptation and geographical spread. Nat Rev Microbiol 2016; 14: 235–50.

[8] van den Beld MJC, Reubsaet FAG. Differentiation between *Shigella*, enteroinvasive *Escherichia coli* (EIEC) and noninvasive *Escherichia coli*. Eur J Clin Microbiol Infect Dis 2012; 31: 899–904.

[9] Yang C, Li P, Zhang X, Ma Q, Cui X, Li H, et al. Molecular characterization and analysis of high-level multidrug-resistance of *Shigella flexneri* serotype 4s strains from China. Sci Rep 2016; 6: 29124.

[10] Pavlovic M, Luze A, Konrad R, Berger A, Sing A, Busch U, et al. Development of a duplex real-time PCR for differentia- tion between *E. coli* and *Shigella* spp. J Appl Microbiol 2011; 110: 1245–51.

[11] Rahi P, Prakash O, Shouche YS. Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) based microbial identifications: challenges and scopes for microbial ecologists. Front Microbiol 2016; 7: 1359.

[12] Løbersli I, Wester AL, Kristiansen Å, Brandal LT. Molecular differentiation of *Shigella* spp. from enteroinvasive *E. coli*. Eur J Microbiol Immunol 2016; 6: 197–205.

[13] Chattaway MA, Schaefer U, Tewolde R, Dallman TJ, Jenkins C. Identification of *Escherichia coli* and *Shigella* species from whole-genome sequences. J Clin Microbiol 2017; 55: 616–23.

[14] Pettengill EA, Pettengill JB, Binet R. Phylogenetic Analyses of *Shigella* and Enteroinvasive *Escherichia coli* for the identification of molecular epidemiological markers: whole-genome comparative analysis does not support distinct genera designation. Front Microbiol 2015; 6: 1573.

[15] Al Bayssari C, Olaitan AO, Dabboussi F, Hamze M, Rolain JM. Emergence of OXA-48-producing *Escherichia coli* clone ST38 in fowl. Antimicrob Agents Chemother 2015; 59: 745–6.

[16] Dhiviya Prabaa MS, Naveen Kumar DR, Yesurajan IF, Anandan S, Kamini W, Balaji V. Identification of non-serotypeable *Shigella* spp. using genome sequencing: a step forward. Future Sci OA 2017; 3: FSO229.

[17] Bélanger SD, Boissinot M, Ménard C, Picard FJ, Bergeron MG. Rapid detection of Shiga toxin-producing bacteria in feces by multiplex PCR with molecular beacons on the smart cycler. J Clin Microbiol 2002; 40: 1436–40.

[18] EUCAST. The European committee on antimicrobial susceptibility testing, http://www.eucast.org/.

[19] Roschanski N, Fischer J, Guerra B, Roesler U. Development of a multiplex real-time PCR for the rapid detection of the predominant beta-lactamase genes CTX-M, SHV, TEM and CIT-type AmpCs in Enterobacteriaceae. PloS One 2014; 9: e100956.

[20] Edelstein M, Pimkin M, Palagin I, Edelstein I, Stratchounski L. Prevalence and molecular epidemiology of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob Agents Chemother 2003; 47: 3724–32.

[21] Tajbakhsh M, Garcia Migura L, Rahbar M, Svendsen CA, Mohammadzadeh M, Zali MR, et al. Antimicrobial-resistant *Shigella* infections from Iran: an overlooked problem?. J Antimicrob Chemother 2012; 67: 1128–33.

[22] Yagi T, Kurokawa H, Shibata N, Shibayama K, Arakawa Y. A preliminary survey of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS Microbiol Lett 2000; 184: 53–6.

[23] Khot PD, Fisher MA. Novel approach for differentiating *Shigella* species and *Escherichia coli* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2013; 51: 3711–6.

[24] Hsu BM, Wu SF, Huang SW, Tseng YJ, Ji DD, Chen JS, et al. Differentiation and identification of *Shigella* spp. and enteroinvasive *Escherichia coli* in environmental waters by a molecular method and biochemical test. Water Res 2010; 44: 949–55.

[25] Ekwall E, Dimander M. Comparison of micro-ID with API 20E for identification of *Salmonella, Shigella* and *Yersinia* species. Eur J Clin Microbiol 1982; 1: 134–7.

[26] Li B, Liu H, Wang W. Multiplex real-time PCR assay for detection of *Escherichia coli* O157:H7 and screening for non-O157 Shiga toxin-producing *E. coli*. BMC Microbiol 2017; 17: 215.

[27] Khot PD, Couturier MR, Wilson A, Croft A, Fisher MA. Optimization of matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis for bacterial identification. J Clin Microbiol 2012; 50: 3845–52.

[28] Kim HJ, Ryu JO, Song JY, Kim HY. Multiplex polymerase chain reaction for identification of *Shigellae* and Four *Shigella* species using novel genetic markers screened by comparative genomics. Foodborne Pathog Dis 2017; 14: 400–6.

[29] Liu B, Knirel YA, Feng L, Perepelov AV, Senchenkova SN, Wang Q, et al. Structure and genetics of *Shigella* O antigens. FEMS Microbiol Rev 2008; 32: 627–53.

[30] Anandan S, Muthuirulandi Sethuvel DP, Gajendiren R, Verghese VP, Walia K, Veeraraghavan B. Molecular characterization of antimicrobial resistance in clinical *Shigella* isolates during 2014 and 2015: trends in South India. Germs 2017; 7: 115–22.

[31] Shakya G, Acharya J, Adhikari S, Rijal N. Shigellosis in Nepal: 13 years review of nationwide surveillance. J Health Popul Nutr 2016; 35: 36.

[32] Devanga Ragupathi NK, Muthuirulandi Sethuvel DP, Inbanathan FY, Veeraraghavan B. Accurate differentiation of *Escherichia coli* and *Shigella* serogroups: challenges and strategies. New Microbes New Infect 2018; 21: 58–62.

[33] Ashida H, Sasakawa C. *Shigella* IpaH family effectors as a versatile model for studying Pathogenic Bacteria. Front Cell Infect Microbiol 2015; 5: 100.

[34] Ashida H, Toyotome T, Nagai T, Sasakawa C. *Shigella* chromosomal IpaH proteins are secreted via the type III secretion system and act as effectors. Mol Microbiol 2007; 63: 680–93.

[35] Hazen TH, Leonard SR, Lampel KA, Lacher DW, Maurelli AT, Rasko DA. Investigating the relatedness of Enteroinvasive *Escherichia coli* to other *E. coli* and *Shigella* isolates by using comparative genomics. Infect Immun 2016; 84: 2362–71.

[36] Kasai H, Tamura T, Harayama S. Intrageneric relationships among Micromonospora species deduced from *gyrB*-based phylogeny and DNA relatedness. Int J Syst Evol Microbiol 2000; 50: 127–34.

[37] Wang LT, Lee FL, Tai CJ, Kasai H. Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. Int J Syst Evol Microbiol 2007; 57: 1846–50.

[38] Vos M, Quince C, Pijl AS, de Hollander M, Kowalchuk GA. A Comparison of *rpoB* and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. PLoS One 2012; 7: e30600.

[39] Chamoun K, Farah M, Araj G, Daoud Z, Moghnieh R, Salameh P, et al. Lebanese Society of Infectious Diseases study group (LSID study group): surveillance of antimicrobial resistance in Lebanese hospitals: retrospective nationwide compiled data. Int J Infect Dis 2016; 46: 64–70.

[40] Daoud Z, Hakime N. Prevalence and susceptibility patterns of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in a general university hospital in Beirut, Lebanon. Rev Esp Quimioter 2003; 16: 233–8.

[41] Keddy KH, Sooka A, Crowther-Gibson P, Quan V, Meiring S, Cohen C, et al. Group for Enteric, Respiratory, and Menin- geal Disease Surveillance in South Africa (GERMS-SA): sys- temic shigellosis in South Africa. Clin Infect Dis 2012; 54: 1448–54.

[42] Madhavan A, Balakrishnan S, Vasudevapanicker J. Antibiotic susceptibility pattern of *Shigella* isolates in a tertiary healthcare center. J Lab Phys 2018; 10: 140–4.

[43] Liu Y, Cheng Y, Yang H, Hu L, Cheng J, Ye Y, et al. Characterization of extended-spectrum b-lactamase genes of *Shigella flexneri* isolates with fosfomycin resistance from patients in China. Ann Lab Med 2017; 37: 415–9.

[44] Dekker JP, Frank KM. *Salmonella, Shigella*, and *Yersinia*. Clin Lab Med 2015; 35: 225–46.

[45] Valenzuela C, Legarraga P, Pena A, Arenas A, Berkowitz L, Ramirez G, et al. Etiologic and clinical characterization of community acquired gastroenteritis in adult patients in a Chilean emergency room by the film array GI panel. PLoS One 2018; 13: e0207850.

Chapter II: Genome sequence of a multidrug-resistant *Campylobacter coli* strain isolated from newborn suffering from severe diarrhea in Lebanon.

Introduction:

Campylobacter is a zoonotic foodborne bacterium that has evolved several mechanisms of AMR among others, enzymatic degradation, antimicrobial target site modification, and active efflux pumps [1, 2]. In Lebanon, the real campylobacteriosis burden is underestimated as the disease is not obligatory notified. Meanwhile, WGS has become a part of the national and international surveillance of foodborne diseases, including *Campylobacter*, in many countries [3–5]. Noteworthy, the quantum leap in clinical laboratories has been made with the advent of high- throughput sequencing platforms able to scale up the WGS for large samples, broaden the range of microorganism detection usually limited by predefined targets with PCR, and decipher many other microbiological aspects never tackled before simultaneously in the same experiment workflow.

According to the aim of the World Health Organization (WHO) to gradually integrate in a smooth manner the WGS technology as a surrogate for the traditional routine pathogen characterization, we performed a WGS in chapter 2 entitled **"Genome sequence of a multidrug-resistant *Campylobacter coli* strain isolated from newborn suffering from severe diarrhea in Lebanon",** to exploit how powerful is this technique to identify, detect antimicrobial resistance determinants, explain disease severity, and to determine the clonal membership of a multidrug-resistant (MDR) *Campylobacter* isolate from 2 months aged newborn in Tripoli-Lebanon.

WGS identified the strain as *C. coli,* which, in turn, hints seemingly for the potential high clinical burden of this species in Lebanon. Additionally, our results revealed the usefulness of WGS to decipher the resistome and virulome in a single workflow. Several genetic determinants along with their genetic backgrounds are identified as responsible for the multidrug resistance phenotype, among them resistance genes and mutations known or described for the first time as potentially involved in resistance. On the other hand, WGS enabled us to track the evolution and the richness of *Campylobacter* plasmidome and this by identifying two plasmids differing slightly from the previously described plasmids (pCCDM33S and pCCDM183). Besides, WGS allowed us to identify for the first time a novel sequence type (ST-9588), which is belonged to the ST-828 clonal complex known to encompass mainly *C. coli* isolates from the chicken and humans [6]. Finally, comparing the genome of our strain with several *C. coli* genomes from NCBI that belonged to different biological sources shed light on the complexity of zoonotic transmissions between the different reservoirs (human, poultry, cattle, swine, and others).

This is the first study considered the use of WGS to characterize a *Campylobacter* clinical isolate in Lebanon. Although the integration of WGS in Lebanon seems elusive at this time, this study highlights how WGS can open new prospects in disease surveillance never achieved before using traditional phenotypic and molecular techniques.

REFERENCES

1. Blaser MJ, Engberg J (2008) Clinical Aspects of *Campylobacter jejuni* and *Campylobacter coli* Infections. In: Blaser MJ, Nachamkin I, Szymanski CM (eds) *Campylobacter*, Third Edition. American Society of Microbiology, pp 99–121
2. Whitehouse CA, Zhao S, Tate H (2018) Antimicrobial Resistance in *Campylobacter* Species: Mechanisms and Genomic Epidemiology. Adv Appl Microbiol 103:1–47. https://doi.org/10.1016/bs.aambs.2018.01.001
3. Allard MW, Strain E, Melka D, et al (2016) Practical Value of Food Pathogen Traceability through Building a Whole-Genome Sequencing Network and Database. J Clin Microbiol 54:1975–1983. https://doi.org/10.1128/JCM.00081-16
4. CFSAN (2019) GenomeTrakr Network. In: FDA. http://www.fda.gov/food/whole-genome-sequencing-wgs-program/genometrakr-network. Accessed 23 Aug 2019
5. WHO (2018) Whole genome sequencing for foodborne disease surveillance: landscape paper. World Health Organization
6. Nohra A, Grinberg A, Midwinter AC, et al (2016) Molecular Epidemiology of *Campylobacter coli* Strains Isolated from Different Sources in New Zealand between 2005 and 2014. Appl Environ Microbiol 82:4363–4370. https://doi.org/10.1128/AEM.00934-16

Article

Genome sequence of a multidrug-resistant *Campylobacter coli* strain isolated from newborn suffering from severe diarrhea in Lebanon.

Fatima Bachir Halimeh[1, 2], Rayane Rafei[1], Seydina Diene[2], Marwan Osman[1], Randa Jamal Akoum[3], Walid Moudani[1], Monzer Hamze[1]*, Jean-Marc Rolain[2]

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[1]Laboratoire Microbiologie Santé et Environnement (LMSE), Ecole Doctorale des Sciences et de Technologie, Faculté de Santé Publique, Université Libanaise, Tripoli, Liban.

[2]Aix Marseille University, IRD, APHM, MEPHI, IHU Méditerranée Infection, Faculté de Médecine et de Pharmacie, 19-21 boulevard Jean Moulin, 13385 Marseille.

[3]Pédiatre, Clinique Jamal et Akoum, Tripoli, Liban.

* Corresponding author

Monzer Hamze: mhamze@monzerhamze.com

Abstract:

A multi-drug resistant (MDR) *Campylobacter coli* strain was isolated from 2-months newborn suffering from severe diarrhea in Lebanon. The aims of the present were to exploit the potential of the whole-genome sequencing (WGS) in determining the genetic basis of the antimicrobial resistance, and disease severity, as well as in understanding the epidemiologic background of the Lebanese isolate. Thus, the single isolate was identified using API Campy, MALDI-TOF and *16S rDNA* gene sequencing. The antimicrobial susceptibility was tested by the disc diffusion method. The WGS was analyzed using the Illumina MiSeq platform. Therefore, the WGS analysis showed that macrolides and quinolones resistance was potentially due to the presence of multiple usual and unusual point mutations in chromosomal antibiotic target sites. However, tetracycline and aminoglycoside resistance were provided by a pTet plasmid. The bla_{OXA-61} gene associated with beta-lactam resistance was detected for the first time in a *C. coli* strain from humans in Lebanon. Furthermore, the genome of this isolate harbored a set of 30 virulence-associated genes. The *in silico* MLST identified the isolate as belonging to a new sequence type (ST-9588), a member of ST-828 complex identified mainly in humans and chicken. The phylogenetic tree based on the core genome single nucleotide polymorphisms of our isolate along with other *C. coli* genomes available in NCBI did not identify any precise biological source. Finally, WGS is an enticing technology unveiling many interesting facets of our MDR and virulent isolate.

Keywords: *Campylobacter coli*; Lebanon; Whole-genome sequencing; Antimicrobial resistance; Virulence.

Introduction:

Campylobacter is a zoonotic foodborne bacterium and one of the leading agents of human gastroenteritis worldwide [1]. According to the World Health Organization (WHO), the estimated incidence of gastroenteritis due to *Campylobacter* ranges between 4.4 and 9.3 per 1000 people [2]. With regard to age groups, *Campylobacter* infections (campylobacteriosis) prevailed in children less than 5 years old [3]. Campylobacteriosis is a self-limiting infection and may not require antimicrobial treatment usually. However, in severe or prolonged infections, antimicrobial therapy is required. Clinically, fluoroquinolones and macrolides are the drugs of choice, but in some cases, tetracycline and gentamicin can be used [4]. Indeed, *Campylobacter* has evolved several mechanisms of antimicrobial resistance among others, enzymatic degradation, modification of the antimicrobial target site, and active efflux pump [5], which narrow the spectrum of available treatment options. *Campylobacter* is highly mutable to fluoroquinolones, in contrast to macrolides where resistance developed slowly [6] Notably, horizontally transferrable *erm*(B) genes encoding the erythromycin resistance methylases have been recently described in *Campylobacter,* which could significantly impact the epidemiology of macrolide resistance [4]. Besides, *Campylobacter* could develop co-resistance to multiple agents such as aminoglycosides, tetracyclines, and β-lactams by expelling them through efflux pumps (as the well-characterized efflux pump CmeABC) [4]. Along with the increasing antimicrobial resistance levels, *Campylobacter* has a complex multifactorial system for colonization and survival during food processing and virulence in humans. Noteworthy, its human colonization capacity and virulence can be enhanced after poultry passage [7].

Whole-Genome Sequencing (WGS) can unravel a surprising level of genomic resolution allowing the identification of isolates as well as of a broad range of genotypic traits, such as virulence genes, antimicrobial determinants, even more, the *Campylobacter* epidemiology and their clonal circulation [8, 9]. The WGS is implemented nowadays as part of the national and international surveillance and response to Foodborne Diseases (FBDs) in many countries. For example, the GenomeTrakr (GT) network in the USA uses the WGS for the traceability of FBDs, among others, *Campylobacter* [10, 11]. Likewise, the WHO aims to implant the WGS in developing countries, but a gradual transition from traditional to WGS-based routine characterization of pathogens is needed to ensure a smooth integration of this technology [12].

In Lebanon, the implementation of WGS in Lebanese routine workflow seems to be elusive currently with the conspicuous deficit of pathogen identification capabilities that are

mostly relied on traditional phenotypic methods. An MDR *Campylobacter* strain was isolated from newborn suffering from severe fever, diarrhea, and abdominal pain in Tripoli, North Lebanon on 20th December 2016. This study aimed to exploit the potential of using WGS in the epidemiologic surveillance of *Campylobacter* spp. particularly in developing countries like Lebanon and how powerful is this technique to identify, to detect antimicrobial resistance determinants, to explain disease severity and to determine the clonal membership of the Lebanese isolate. To the best of our knowledge, this is the first study considered the use of WGS to characterize a *Campylobacter* clinical isolate in Lebanon.

Materials and Methods:

Identification and antimicrobial susceptibility testing.

The isolate (CMUL 139 or also known as *C. coli* Lebanon_2016) was cultivated on BD Columbia Agar with 5% Sheep Blood (Becton Dickinson GmbH, Heidelberg, Germany) under microaerophilic conditions using atmospheric generators (GENbox Microaer, bioMérieux, France). Identification was performed using API® Campy (BioMérieux, Marcy-l'Etoile, France), followed by MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry) (Bruker, Massachusetts, United States) and *16S rDNA* gene sequencing [13]. *In vitro* antimicrobial susceptibility testing by the disc diffusion method was carried out using a large spectrum of antibiotics (30 antibiotics). For ampicillin, amoxicillin/ clavulanic acid, erythromycin, ciprofloxacin, doxycycline, ertapenem, and gentamicin, the results were interpreted according to 2016 EUCAST recommendations for *Campylobacter* [14]. For other antibiotics, amoxicillin, cefotaxime, ceftriaxone, cefoxitin, aztreonam, cephalothin, ticarcillin, ticarcillin/clavulanic acid, imipenem, amikacin, tobramycin, kanamycin, nalidixic acid, fosfomycin, nitrofuran, and colistin, the interpretation was done according to EUCAST recommendations for *Enterobacteriaceae*. We also tested oxacillin, streptomycin, spectinomycin, pristinamycin, rifampicin, clindamycin, and linezolid.

Whole-genome sequencing and analysis.

Genomic DNA was extracted using EZ1 DNA extraction kits (Qiagen NV, Venlo, the Netherlands) with the EZ1 Advanced XL biorobot according to the manufacturer's instructions. The whole genome of the isolate was paired-end sequenced using the MiSeq platform (Illumina, Inc., San Diego, CA, USA). Raw reads were assembled using the Edena assembly [15]. The isolate is definitely identified using the CVTree 3 software [16]. The genome was annotated with Rapid Prokaryotic Genome Annotation software (PROKKA) [17]. Analysis of virulome and resistome of the studied genome (*de novo* assemblies of the newly sequenced isolate as well as some publicly available genomes) was performed using the last

version of ABRicate (https://usegalaxy.org.au/) based on a BLAST search against the Virulence Factors Database (VFDB) and the Comprehensive Antibiotic Resistance Database (CARD) respectively. To detect the presence of plasmids harbored by this isolate, the software PlasmidSeeker was used [18]. Moreover, Circos software was used to represent circularly the plasmid sequence [19].

The epidemiological source investigation of our isolate was achieved by collecting 17 *C. coli* draft genomes and 20 *C. coli* complete genome, belonging to different sources, from NCBI (https://www.ncbi.nlm.nih.gov/genome/) (Annex 1), then by comparing the core-genome of all included isolates using Parsnp tools [20]. The program Parsnp calls single nucleotide polymorphism (SNP) through building a core genome alignment. The *C. coli* strain OR12 (Accession Number: NZ_CP013733.1) was used as a reference genome. The called SNPs were filtered first using the vcfutils.pl varFilter with default parameters, then by vcftools to remove SNPs within 100bp as well as indels. Furthermore, the filtered core genome alignment was inputted in Gubbins to remove regions of recombination and to simultaneously build a maximum likelihood tree using RAxML [21]. Fig tree software was used to display graphically the tree generated by Gubbins [22]. Multi-Locus Sequence Typing (MLST) was performed *in silico* using the WGS data according to the *Campylobacter* PubMLST scheme. The newly identified sequence type was coded by PubMLST (https://pubmlst.org/). The draft genome sequence of *C. coli* CMUL 139 has been deposited in GenBank under the following accession number VTWZ00000000.1 and BioProject PRJNA562140.

Results:

C. coli Lebanon_2016 strain was isolated from a 2-months newborn with a clinical table of abdominal pain, diarrhea, and fever. He was treated by Klacid® (clarithromycin) and he is recovered. The baby was born by cesarean delivery and fed by his maternal milk. The draft genome sequence of this isolate consisted of 111 contigs with an N50 length of 48.73 Kbp and a genome coverage of 47.6x. The genome size was 1.83 Mbp, comparable to other published genomes of *C. coli* ranging from 1.71 Mbp to 2.03 Mbp [23, 24]. The GC content was 30.1% similar to previously characterized *C. coli* genomes [23, 24]. The draft genome of *C. coli* Lebanon_2016 contains 1886 coding sequences with 2 rRNA and 38 tRNA.

Before the WGS, different identities were assigned to this isolate. Api® Campy determined the isolate as belonging to *Campylobacter* genus, while MALDI-TOF MS identified it as *C. coli* (score 2.43) and the *16S rDNA* sequencing defined it as *C. coli* with 99.5% identity. Otherwise, WGS confirmed the *16S rDNA* sequencing identification where our isolate clustered clearly with *C. coli* genomes using CVTree (Annex 2).

Roughly, two plasmids were identified in *C. coli* Lebanon_2016. PlasmidSeeker determined the presence of a conjugative plasmid sized 27,104 bp with 34 coding sequences (CDS) encoding for the type IV secretion system responsible for the bacterial horizontal genes transfer by contact-dependent manner called conjugation and sharing 85% similarity with the previously described *C. coli* plasmid pCCDM33S. Four additional hypothetical proteins differed our identified plasmid from the previously described one (Fig. 1-a). The second plasmid shared a great homology with *C. coli* plasmid pCCDM183. However, different from the previously described plasmid sized 55,122 bp, the identified plasmid has a superior size (65,968 bp) and was distributed on 6 contigs (Fig. 1-b). Notably, the added fragment (10,846 bp) in the identified plasmid coded for 15 hypothetical proteins, one conjugal gene (DNA primase TraC) and Lactococcin-G-processing and transport ATP-binding protein LagD. The plasmid also carried the conjugation machinery components with the *tet*O gene conferring the tetracycline resistance, as well as, *aph*7, *aph*3-III, *aph*2-Ib, *aac*6-Im, *ant*6-Ia and *sat*4A genes conferring the aminoglycoside resistance.

Fig. 1: Circular representation for the two plasmids held by *C. coli* Lebanon_2016 isolate. a: The whole plasmid is found on one contig (green line). b: The plasmid is divided on 6 contigs (brown, dark red, army, red, green and grey lines). Circos software was used to circularly represent the plasmids and figuring out the alignment between the reference plasmids and *C. coli* Lebanon_2016 plasmids.

Of 30 tested antibiotics, *C. coli* Lebanon_2016 was resistant to amoxicillin, ampicillin, cefotaxime, ceftriaxone, cefoxitin, aztreonam, cephalothin, ticarcillin, ertapenem, gentamicin, amikacin, tobramycin, kanamycin, nalidixic acid, ciprofloxacin, erythromycin, clindamycin, linezolid, spectinomycin, pristinamycin, rifampicin, fosfomycin, doxycycline, and trimethoprim/sulfamethoxazole instead it was susceptible to amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, imipenem, streptomycin, nitrofuran, and colistin. Several acquired resistance genes located either chromosomally or on plasmids as well as mutations on naturally existing genes were identified as potentially responsible for the observed antimicrobial resistance phenotype (Table 1).

Table *1*: The phenotypic and genotypic antibiotic resistance profiles of *C. coli* Lebanon_2016.

Category	Profile	Genes	Potential Mutation

Beta-lactams	AMP, AMX, CTX, CRO, FOX, ATM, KF, TIC, ERT	*bla*$_{OXA-61}$*	
Tetracycline	DOX	*tet*O**	
Aminoglycoside	GEN, TOB, SPT, AMK, KAN	*aadE***	
		*aad9***	
		*aphA-3***	
		*aph2-Ib***	
		*aac6-Im***	
Macrolide /MLS$_B$	E, CLI, PT	23S rRNA*	A2059G
			A2071G$^+$
		50S ribosomal protein L22*	A193G$^+$, T195G$^+$
			C221G$^+$
			G325A$^+$
			A332C$^+$, G333A$^+$
			A340G$^+$
			G355A$^+$
Fluoroquinolone	NA, CIP	*gyr*A*	C257T
		*gyr*B*	Silent

ND: not detected. MLS$_B$: Macrolides- Lincosamides- Streptogramins B.AMP: Ampicillin, AMX: Amoxicillin, CTX: Cefotaxime, CRO: Ceftriaxone, FOX: Cefoxitin, ATM: Azteronam, KF: Cephalothin, TIC: Ticarcillin, ERT: Ertapenem, DOX: Doxycyclin, GEN: Gentamicin, TOB: Tobramycin, SPT: Spectinomycin, AMK: Amikacin, KAN: Kanamycin, E: Erythromycin, CLI: Clindamycin, PT: Pristinamycin, NA: Nalidixic Acid, CIP: Ciprofloxacin, FOS: Fosfomycin. *: Chromosomic, **: Plasmidic. $^+$: Unusual mutations, Blue: *E. coli* numbering, Red: *C. coli* OR12 numbering.

Virulence factors database (VFDB) in ABRicate revealed the presence of a couple of virulence factors owned by our isolate. These virulence factors are divided into different types based on their roles in bacterial colonization (Table 2).

Table 2: Potential virulence factors harbored by *C. coli* Lebanon-2016 isolate and their pathogenic roles.

Virulence role	**Identified virulence factors encoding genes**	**Roles**
Invasion	*flhA, flhB, fliQ, flip, fliR,*	Components of flagellar T3SS

	flaC	Protein secreted into host cell essential for colonization and invasion
	ciaB	Involved in adhesion
	ciaC	Protein required for full invasion of INT-407 cells
Adhesion	*cadF*	Outer membrane protein
Chemotaxis	*cheA, cheB, cheV, chew*	Chemotaxis protein
Motility	*flaA, flab*	Major flagellin protein
	fliF	Hook-basal body protein
	fliM, fliY	Flagellar motor protein
	flgI	P-ring in the peptidoglycan
	flgH	L ring in the outer membrane
	fliK, flgE	Minor hook components
Toxin	*cdtA, cdtB*	Cytolethal distending toxin subunits
Capsular polysaccharide	*kspM*	Transport gene M
	kspD	Export system periplasmic protein
	kspS	Modification protein
	kspT	Export ATP-binding protein
	kspE	Export system inner membrane protein
	kspF	D-arabinose 5-phosphate isomerase

Regarding MLST analysis, *C. coli* Lebanon_2016 strain was assigned to a new Sequence Type ST-9588, which is belonged to the ST-828 complex, by PubMLST. To understand the phylogenetic relationship of our isolate, core genome SNPs were extracted from 17 draft genomes of *C. coli* and 20 complete genomes (Annex 1). Based on the SNP based phylogenetic tree, it has been demonstrated that *C. coli* Lebanon_2016 did not belong to any specific cluster (Fig. 2). Indeed, it clustered with different *Campylobacter* strains originated from diverse biological sources (poultry, human, and cattle).

Fig. 2: Maximum likelihood phylogenetic tree based on SNPs analysis from the core genome alignment of *C. coli* Lebanon_2016 with NCBI referenced complete and draft genomes, after removing the recombination regions using Gubbins. CC_OR12 was the reference genome.

FigTree V1.4 was used to represent the phylogenetic tree. Paint.Net V4.1.5 was used to represent biological and geographical data. The accession numbers of NCBI strains used in this analysis are shown in **annex 1**.

Discussion:

In Lebanon, the prevalence of campylobacteriosis is profoundly underestimated and few works tackled the occurrence of *Campylobacter* in clinical and poultry sectors. As revealed by these scant studies, the prevalence of *Campylobacter* in humans fluctuated from low (0.7%) [25] to moderate scale (11% to 17%) [26–28], while the prevalence of *Campylobacter* in broiler chicken varied from 67% to 95% [6, 28, 29]. Otherwise, the herein isolation of *C. coli* from newborn suffering severe diarrhea may hint seemingly to the degree of mitigation of the current campylobacteriosis burden in this country. Comparing other countries with Lebanon, the prevalence of *Campylobacter* in reported hospitalized infections in the USA (20%), EU (27.6%), Qatar (25%), and Egypt (25.3%) surpassed that in Lebanon due possibly to the lack of its systematic screening in our country [30–33]. Additionally, among *Campylobacter* species, *C. jejuni* is the main species recovered from human campylobacteriosis and poultry sector in many countries over the world [12, 30]. In Lebanon, the situation differed wherein *C. coli* predominated in both clinical and poultry sector [6, 28] and the identification of *C. coli* herein from a newborn can be an additional proof of its level of involvement in human campylobacteriosis and the extent of potential zoonotic transmissions that could occur between Lebanese humans and poultry.

The identification of *C. coli* Lebanon_2016 was somewhat fastidious. The stool culture is the gold standard to identify *Campylobacter* genus, while species identification is not usually done unless in case of epidemiologic purposes [32]. Nowadays, the phenotypic methods are replaced by genotypic ones that are more accurate, rapid, and have higher discrimination power. We used the *16S rDNA* gene sequencing to identify our strain. However, while *16S rDNA* gene sequencing is extensively used to identify *Campylobacter*, it cannot differentiate between very closely related species, such as *C. jejuni* and *C. coli* [34, 35]. For this, we complemented our identification by the genome sequencing followed by CVTree analysis that closely clustered our strain with another *C. coli* isolates.

The WGS is increasingly becoming a pillar in many surveillance networks across the world as it provides the highest insights into microbial subtyping and ensures a quick turnaround time to detect, understand and intervene in case of outbreaks [12]. Additionally, it can disclose the entire genetic information allowing the community to address many questions

in one test that are until now answered through multiple tests. One of the potential features of WGS is its ability to predict the phenotypic drug resistance tested with the disk diffusion method through the detection of chromosomal and plasmidic resistance genes. Our strain was MDR to several antibiotic classes particularly fluoroquinolones, macrolides, tetracyclines, and aminoglycosides. The term MDR is assigned to a particular *Campylobacter* isolate when presenting resistance to three or more antimicrobial groups (quinolones, macrolides, tetracycline, β-lactam, and chloramphenicol) [36]. The incidence of *Campylobacter* MDR is somehow variable between countries, reaching up 46% and 81%, in Tanzania and Iran, respectively [37, 38]. Recently, a noticeable level of antimicrobial resistance was recorded in Lebanon where 97%, 89%, 74% and 79% of *C. coli* isolates from broilers at Lebanese slaughterhouses were resistant to ciprofloxacin, amoxicillin, erythromycin, and gentamicin, respectively [6]. Noteworthy, the extensive uncontrolled use of antibiotics in veterinary livestock in Lebanon might explain the high isolation of MDR strains in the poultry sector [6] and subsequently the emergence of such strains in humans. Comparing the ability to acquire multidrug resistance between *C. coli* and *jejuni* species, *C. coli* has the potential to develop more fluoroquinolones and macrolides resistance than *C. jejuni* [6, 39]. The potential mechanisms of phenotypic antimicrobial resistance observed in our strain were investigated for genetic patterns known to be associated with resistance. The observed fluoroquinolone resistance in *C. coli* Lebanon_2016 can be linked to C257T *gyrA* mutation, which leads to the T86I substitution in GyrA. This notorious single point mutation is sufficient to reduce the susceptibility towards fluoroquinolones [4]. Notably, *C. coli* Lebanon_2016 has several synonymous mutations on *the gyrB* gene. However, to date, mutations in *gyrB* have not been associated with fluoroquinolone resistance in *Campylobacter* species [40]. Moreover, the constitutive MLS_B resistance phenotype (cross-resistance to macrolides, lincosamides, streptogramin b) could be explained by the observed mutation A2059G on the 23S rRNA within the Domain V, which was considered the major mechanism [41]. Additionally, other potential unusual mutations are observed in the 23S rRNA (A2071G) and the ribosomal protein L22, however, any mutation is detected on the ribosomal protein L4 [4]. Although the *erm* genes were the common determinants responsible for the MLS_B phenotype in other genera like Gram-positive bacteria [42], they are considered rare in *Campylobacter* genus and are recently described in *C. coli* isolates from chicken and swine in China and Spain in 2014-2016 [5, 43, 44]. *C. coli* Lebanon_2016 lacked the *erm*(B) gene, even the last was increasingly reported in *Campylobacter* strains notably within the MDRGI (Multidrug Resistance Gene island). In addition, the presence of bla_{OXA-61} gene in the *C. coli* Lebanon_2016 chromosome can explain

the β-lactam resistance phenotype. The *bla*$_{OXA-61}$ gene is one of the *Campylobacter* β-lactam resistance mechanisms primarily observed in *C. jejuni*. Recently, *C. coli* strains harboring *bla*$_{OXA-61}$ were isolated from pigs [45, 46]. To the best of our knowledge, there is the first report of *bla*$_{OXA-61}$ in *C. coli* isolates from humans in Lebanon. Additionally, *C. coli* Lebanon_2016 harbored the most predominant antibiotic efflux pump (CmeABC), which belonged to resistance-nodulation cell division superfamily of multidrug efflux transporters, and functioned synergistically with other mechanisms in conferring high-level resistance to quinolones, macrolides, and β-lactam [4, 47, 48]. In order to explain the decreased susceptibility to linezolid, we looked for the presence of some acquired linezolid resistance determinants (*cfr*, *cfr*(C), *cfr*(B), *poxt*(A) and *optr*(A)) or some known mutations that affect mostly 23S rRNA and 50S ribosomal proteins L3, L4 and L22 in Gram-positive bacteria [49, 50]. However, any of these mechanisms were detected in our strain and the observed resistance may be referred to unusual potential mutations not described before in 23S rRNA or to the presence of the efflux pump (CmeABC efflux pump). Remarkably, among acquired linezolid resistance determinants, only the *cfr*(C) gene located in a conjugative pTet MDR/virulence plasmid was described in florfenicol resistant *C. coli* isolates from cattle but not in humans and was liable for PhLOPSA resistance phenotype (resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramins) [51, 52].

Moreover, the WGS revealed that *C. coli* Lebanon_2016 contained 2 plasmids similar to those previously described in retail chicken liver strains [53]. The first plasmid was similar to that firstly described in *C. coli* isolate from the chicken liver (pCCDM33S), which belonged to type 2 *C. coli* specific plasmids. Our first plasmid shared with type 2 plasmids most of the core genes as the conjugation machinery components (Type IV secretion system protein VirB1, single-stranded DNA-binding protein, protein TraI, and protein TraL) and had a size within the size range of type 2 (24 to 32 kb) [54]. The second identified plasmid was similar to the pCCDM183 plasmid firstly detected in *C. coli* isolate from chicken liver and which belonged to type 1 *Campylobacter* plasmids (pTet plasmids) [53]. These pTet plasmids are commonly found in *C. coli* and *C. jejuni* and carried important resistance and virulence genes. Like other pTet plasmids, our second plasmid had a size greater than 48Kb and contained genes encoding for TetO (a key core gene of pTet plasmids), type IV secretion system proteins as VirB4, VirB8, VirB9, VirB10, VirB11, protein VirD4, single-stranded DNA-binding protein, and virulence-associated protein 2 (VapD) [54]. In addition to these genes, our second plasmid carried an arsenal of interesting elements. For example, a lactococcin (bacteriocin) processing and transport ATP-binding protein sharing a high identity with that described in *Campylobacter*

helveticus together with a conjugal gene were found as a special signature of this plasmid, not present in the previously described one (pCCDM183). Lactococcin bacteriocins are mainly produced by *Lactococcus lactis* [55, 56]. Our second plasmid can acquire this bacteriocin transporter through conjugation either from *C. helveticus* or within the neonate intestine where many lactic acid bacteria thrive. Noteworthy, usually the bacteriocin transporter gene is found together with the bacteriocin gene within the same cluster. However, the sole detection of the transporter in this plasmid can be explained either by the sole acquisition of transporter or by the deletion of the bacteriocin gene after its acquisition due to the absence of any competitive advantage.

It's noteworthy to mention that the second plasmid also harbored a cluster of antimicrobial resistance genes encoding resistance for tetracycline (*tet*O), aminoglycosides (*aphA-3, aadK, sat4, aph2-Ib and aac6-Im*) and hygromycin (*hph*) (Fig. 3). This cluster can be classified as an island as having a high GC% relative to the plasmidic background (36.83% Vs. 28.34%), and a size greater than 10 kb [57]. This island can be acquired from an unknown source through conjugation and then evolved by multiple events of recombination. A recent genomic island known as MDRGI was first identified in *C. coli* isolate from broiler chicken and then multiple MDRGI variants were detected throughout the world [58]. MDRGIs, which usually had a chromosomal genetic background, had several resistance genes as *erm*(B) and those encoding for tetracyclines, aminoglycosides and fosfomycin resistance [58]. Therefore, the identified resistance gene cluster herein distinguished from other previously reported MDRGI islands (only transferable under laboratory conditions) by being plasmidic and subsequently naturally transferable [58]. This feature leads to spread horizontally resistance across bacteria with bad consequences on the treatment outcome. The other distinctive features of our cluster are the ability to confer only resistance to tetracyclines (*tetO*) and aminoglycosides (*aphA-3, aadK, sat4, aph2-Ib and aac6-Im*) not macrolides as *erm*(B) gene was absent (Fig. 3) [59].

Fig. 3: Plasmidic organization of multidrug resistance genes clusters harbored by *C. coli* Lebanon_2016 (carried on the second identified plasmid), *C. coli* CVM N29710 (accession number: CP004067), *C. jejuni* T1-21 (accession number: CP013117) and *C. coli* BP3183 (accession number: CP017872) strains. Aminoglycoside resistance genes are in blue, tetracycline resistance gene (*tetO*) is in green, streptothricin resistance gene (*sat4*) is in red, hygromycin resistance gene (*hph*) is in yellow and genes coding for hypothetical proteins are in gray.

Comparing our multidrug resistance cluster to previously identified plasmidic clusters, it was similar to *C. coli* from the chicken liver (*C. coli* BP3183 pCCDM183) with some unique resistance genes (*aph2-Ib* and *aac6-Im*) [53]. Recently, *C. coli* BP3183 pCCDM183 resistance cluster was similarly found in *C. jejuni* from retails chicken (*C. jejuni* T2-21 megaplasmid) where the two differed by two hypothetical encoding genes and one aminoglycoside resistance gene (Fig. 3) [60]. It has also been demonstrated that *C. jejuni* T2-21 megaplasmid resistance cluster shared similarity with that firstly described in gentamicin resistant *C. coli* isolate from retails chicken (*C. coli* CVM pN92710-1), which was developed from ancestor plasmid (pTet plasmid) known to carry tetracycline resistance genes [61] (Fig. 3). Therefore, more thoroughly studies must be carried out to untangle the plasmidome of *Campylobacter* genus, trace their evolution, as well as track their circulation in resistant strains [54].

A bunch of virulence factors was also identified in *C. coli* Lebanon_2016 (Table 2) that are responsible for the host colonization [62]. Along with the predisposing host factors, these virulence factors are required for symptomology and disease severity [7]. Noteworthy, *C. coli* Lebanon_2016 held 2 out of three genes encoding for Cytolethal Distending Toxin (CDT), which can be secreted by multiple pathogenic Gram-negative bacteria. In *Campylobacter*, CDT provides the ability of the host immune system escaping, thus leading to long-term colonization. It also promotes inflammation by inducing inflammatory chemokines/cytokines production [63–65]. The presence of all three genes is required for the toxin to be functionally active [7]. It has been suggested that the truncated gene in the *Campylobacter* CDT cluster can come from genomic rearrangements through recombination or mutation or as an evolving act to persist in the environment [66]. Moreover, our isolate carried a set of genes involved in adhesion, invasion, colonization, and chemiotaxis, which can be considered paramount for disease severity. Indeed, mutation experiments affecting these genes showed reduced adhesion and invasion, enhancing thus the disease elimination [7].

Our comparative genomics analysis of *C. coli* Lebanon_2016 with 37 *C. coli* genomes available at NCBI, suggested that our strain did not belong to any specific cluster (Fig. 1). Furthermore, the 37 genomes are intermixed across the tree regardless of their biological sources, which can highlight the complexity of zoonotic transmission pathways between the different sources. This is consistent with other studies revealing that *Campylobacter* is not very clonal and evolves by the horizontal transmission of large DNA segments that can even occur between two different *Campylobacter* species as *C. coli* and *C. jejuni* [67, 68]. The MLST showed that the strain belonged to clonal complex ST-828, which mainly contains *C. coli* isolates recovered from chicken and humans [69]. The source of newborn infection appeared

ambiguous and many hypotheses could be suggested. The baby could be infected: through lack of maternal hygiene, after homemade food processing; after direct contact with domicile animal not present in the family house; by indirect transmission through having ridden in a shopping car next to poultry products [70]; or by maternal consumption of chicken prepared in a restaurant followed by lack of hygiene of baby's breastfeeding [71].

Conclusion:

In summary, WGS appears to be a very promising technology giving a quick snapshot of the isolate identity, the nature of antimicrobial and virulence determinants harbored by the isolate, even more, the subtyping insights into the epidemiological source of isolate. However, before a quick implementation of WGS in our country, many deficiencies related to pathogen screening, diagnostic, and notification should be considered and plugged.

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Conflict of interest

None to declare

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References

1. Blaser MJ, Engberg J (2008) Clinical Aspects of *Campylobacter jejuni* and *Campylobacter coli* Infections. In: Blaser MJ, Nachamkin I, Szymanski CM (eds) *Campylobacter*, Third Edition. American Society of Microbiology, pp 99–121
2. WHO, FAO, WOAH (2013) the global view of *Campylobacter* report of an expert consultaion, Utrecht, Netherlands, 9-11 July 2012. Utrecht, Netherlands.
3. Kaakoush NO, Castaño-Rodríguez N, Mitchell HM, Man SM (2015) Global Epidemiology of *Campylobacter* Infection. Clin Microbiol Rev 28:687–720. https://doi.org/10.1128/CMR.00006-15
4. Shen Z, Wang Y, Zhang Q, Shen J (2018) Antimicrobial Resistance in *Campylobacter* spp. In: Antimicrobial Resistance in Bacteria from Livestock and Companion Animals. American Society of Microbiology, pp 317–330
5. Whitehouse CA, Zhao S, Tate H (2018) Antimicrobial Resistance in *Campylobacter* Species: Mechanisms and Genomic Epidemiology. Adv Appl Microbiol 103:1–47. https://doi.org/10.1016/bs.aambs.2018.01.001
6. Rafei R, Al Kassaa I, Osman M, et al (2019) Molecular epidemiology of *Campylobacter* isolates from broiler slaughterhouses in Tripoli, North of Lebanon. Br Poult Sci. https://doi.org/10.1080/00071668.2019.1645945
7. Bolton DJ (2015) *Campylobacter* virulence and survival factors. Food Microbiol 48:99–108. https://doi.org/10.1016/j.fm.2014.11.017
8. Pfaller MA (1999) Molecular epidemiology in the care of patients. Arch Pathol Lab Med 123:1007–1010. https://doi.org/10.1043/0003-9985(1999)123<1007:MEITCO>2.0.CO;2
9. Zhao S, Tyson GH, Chen Y, et al (2016) Whole-Genome Sequencing Analysis Accurately Predicts Antimicrobial Resistance Phenotypes in *Campylobacter* spp. Appl Environ Microbiol 82:459–466. https://doi.org/10.1128/AEM.02873-15
10. Allard MW, Strain E, Melka D, et al (2016) Practical Value of Food Pathogen Traceability through Building a Whole-Genome Sequencing Network and Database. J Clin Microbiol 54:1975–1983. https://doi.org/10.1128/JCM.00081-16
11. CFSAN (2019) GenomeTrakr Network. In: FDA. http://www.fda.gov/food/whole-genome-sequencing-wgs-program/genometrakr-network. Accessed 23 Aug 2019
12. WHO (2018) Whole genome sequencing for foodborne disease surveillance: landscape paper. World Health Organization
13. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703.

https://doi.org/10.1128/jb.173.2.697-703.1991

14. EUCAST (2016) The European Committee on Antimicrobial Susceptibility Testing. http://www.eucast.org/. Accessed 1 Feb 2019

15. Hernandez D, Tewhey R, Veyrieras J-B, et al (2014) De novo finished 2.8 Mbp *Staphylococcus aureus* genome assembly from 100 bp short and long range paired-end reads. Bioinformatics 30:40–49. https://doi.org/10.1093/bioinformatics/btt590

16. Gao L, Qi J, Sun J, Hao B (2007) Prokaryote phylogeny meets taxonomy: an exhaustive comparison of composition vector trees with systematic bacteriology. Sci China C Life Sci 50:587–599. https://doi.org/10.1007/s11427-007-0084-3

17. Seemann T (2014) Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153

18. Roosaare M, Puustusmaa M, Möls M, et al (2018) PlasmidSeeker: identification of known plasmids from bacterial whole genome sequencing reads. PeerJ 6:e4588. https://doi.org/10.7717/peerj.4588

19. Krzywinski M, Schein J, Birol İ, et al (2009) Circos: An information aesthetic for comparative genomics. Genome Res 19:1639–1645. https://doi.org/10.1101/gr.092759.109

20. Treangen TJ, Ondov BD, Koren S, Phillippy AM (2014) The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. 15:

21. Croucher NJ, Page AJ, Connor TR, et al (2015) Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res 43:e15. https://doi.org/10.1093/nar/gku1196

22. Rambaut A (2007) FigTree. http://tree.bio.ed.ac.uk/software/. Accessed 23 Aug 2019

23. Ghatak S, He Y, Reed S, et al (2017) Whole genome sequencing and analysis of *Campylobacter coli* YH502 from retail chicken reveals a plasmid-borne type VI secretion system. Genomics Data 11:128–131. https://doi.org/10.1016/j.gdata.2017.02.005

24. O'Kane PM, Connerton IF (2017) Characterisation of Aerotolerant Forms of a Robust Chicken Colonizing *Campylobacter coli.* Front Microbiol 8:513. https://doi.org/10.3389/fmicb.2017.00513

25. Talhouk RS, el-Dana RA, Araj GF, et al (1998) Prevalence, antimicrobial susceptibility and molecular characterization of *Campylobacter* isolates recovered from humans and poultry in Lebanon. J Med Liban 46:310–316

26. Dabboussi F, Alam S, Mallat H, et al (2012) [Preliminary study on the prevalence of *Campylobacter* in childhood diarrhoea in north Lebanon]. East Mediterr Health J Rev Sante Mediterr Orient Al-Majallah Al-Sihhiyah Li-Sharq Al-Mutawassit 18:1225–1228

27. Fadlallah SM, Shehab M, Cheaito K, et al (2019) PulseNet Lebanon: An Overview of Its Activities, Outbreak Investigations, and Challenges. Foodborne Pathog Dis 16:498–503. https://doi.org/10.1089/fpd.2018.2581

28. Greige S, Rivoal K, Osman M, et al (2019) Prevalence and genetic diversity of *Campylobacter* spp. in the production chain of broiler chickens in Lebanon and its association with the intestinal protozoan Blastocystis sp. Poult Sci. https://doi.org/10.3382/ps/pez286

29. Semaan EH, Dib H, Mrad R, et al (2014) Dynamic of *Campylobacter* Species Contamination Along a Poultry Slaughtering Chain. Ital J Food Saf 3:. https://doi.org/10.4081/ijfs.2014.2246

30. EFSA and ECDC (2018) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J 16:e05500. https://doi.org/10.2903/j.efsa.2018.5500

31. El-Tras WF, Holt HR, Tayel AA, El-Kady NN (2015) *Campylobacter* infections in children exposed to infected backyard poultry in Egypt. Epidemiol Infect 143:308–315. https://doi.org/10.1017/S095026881400096X

32. Same RG, Tamma PD (2018) *Campylobacter* Infections in Children. Pediatr Rev 39:533–541. https://doi.org/10.1542/pir.2017-0285

33. Weam B, Abraham M, Doiphode S, et al (2016) Foodborne Bacterial Pathogens Associated with the Risk of Gastroenteritis in the State of Qatar. Int J Health Sci 10:197–207

34. Kulkarni SP, Lever S, Logan JMJ, et al (2002) Detection of *Campylobacter* species: a comparison of culture and polymerase chain reaction based methods. J Clin Pathol 55:749–753. https://doi.org/10.1136/jcp.55.10.749

35. On SL (2001) Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: current status, future prospects and immediate concerns. Symp Ser Soc Appl Microbiol 1S-15S

36. Hakanen AJ, Lehtopolku M, Siitonen A, et al (2003) Multidrug resistance in *Campylobacter jejuni* strains collected from Finnish patients during 1995-2000. J Antimicrob Chemother 52:1035–1039. https://doi.org/10.1093/jac/dkg489

37. Kashoma IP, Kassem II, John J, et al (2016) Prevalence and Antimicrobial Resistance of *Campylobacter* Isolated from Dressed Beef Carcasses and Raw Milk in Tanzania. Microb Drug Resist Larchmt N 22:40–52. https://doi.org/10.1089/mdr.2015.0079

38. Raeisi M, Khoshbakht R, Ghaemi EA, et al (2017) Antimicrobial Resistance and Virulence-Associated Genes of *Campylobacter* spp. Isolated from Raw Milk, Fish, Poultry, and Red Meat. Microb Drug Resist Larchmt N 23:925–933.

https://doi.org/10.1089/mdr.2016.0183

39. CDC (2019) National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) 2015 Human Isolates Surveillance Report | NARMS | CDC. Department of Health and Human Services, Atlanta, Georgia: U.S.

40. Piddock LJV, Ricci V, Pumbwe L, et al (2003) Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. J Antimicrob Chemother 51:19–26. https://doi.org/10.1093/jac/dkg033

41. Corcoran D, Quinn T, Cotter L, Fanning S (2006) An investigation of the molecular mechanisms contributing to high-level erythromycin resistance in *Campylobacter*. Int J Antimicrob Agents 27:40–45. https://doi.org/10.1016/j.ijantimicag.2005.08.019

42. Leclercq R (2002) Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. Clin Infect Dis 34:482–492. https://doi.org/10.1086/324626

43. Qin S, Wang Y, Zhang Q, et al (2014) Report of ribosomal RNA methylase gene erm(B) in multidrug-resistant *Campylobacter coli*. J Antimicrob Chemother 69:964–968. https://doi.org/10.1093/jac/dkt492

44. Florez-Cuadrado D, Ugarte-Ruiz M, Quesada A, et al (2016) Description of an erm(B)-carrying *Campylobacter coli* isolate in Europe. J Antimicrob Chemother 71:841–843. https://doi.org/10.1093/jac/dkv383

45. Obeng AS, Rickard H, Sexton M, et al (2012) Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. J Appl Microbiol 113:294–307. https://doi.org/10.1111/j.1365-2672.2012.05354.x

46. Juntunen P, Heiska H, Hänninen M-L (2012) *Campylobacter coli* isolates from Finnish farrowing farms using aminopenicillins: high prevalence of bla(OXA-61) and β-lactamase production, but low MIC values. Foodborne Pathog Dis 9:902–906. https://doi.org/10.1089/fpd.2012.1176

47. Corcoran D, Quinn T, Cotter L, et al (2005) Characterization of a cmeABC operon in a quinolone-resistant *Campylobacter coli* isolate of Irish origin. Microb Drug Resist Larchmt N 11:303–308. https://doi.org/10.1089/mdr.2005.11.303

48. Cagliero C, Mouline C, Payot S, Cloeckaert A (2005) Involvement of the CmeABC efflux pump in the macrolide resistance of *Campylobacter coli*. J Antimicrob Chemother 56:948–950. https://doi.org/10.1093/jac/dki292

49. Mendes RE, Deshpande LM, Jones RN (2014) Linezolid update: stable in vitro activity following more than a decade of clinical use and summary of associated resistance

mechanisms. Drug Resist Updat Rev Comment Antimicrob Anticancer Chemother 17:1–12. https://doi.org/10.1016/j.drup.2014.04.002

50. Sadowy E (2018) Linezolid resistance genes and genetic elements enhancing their dissemination in enterococci and streptococci. Plasmid 99:89–98. https://doi.org/10.1016/j.plasmid.2018.09.011

51. Tang Y, Dai L, Sahin O, et al (2017) Emergence of a plasmid-borne multidrug resistance gene cfr(C) in foodborne pathogen *Campylobacter*. J Antimicrob Chemother 72:1581–1588. https://doi.org/10.1093/jac/dkx023

52. Zhao S, Mukherjee S, Hsu C-H, et al (2019) Genomic Analysis of Emerging Florfenicol-Resistant *Campylobacter coli* Isolated from the Cecal Contents of Cattle in the United States. mSphere 4:. https://doi.org/10.1128/mSphere.00367-19

53. Marasini D, Fakhr MK (2017) Complete Genome Sequences of Plasmid-Bearing *Campylobacter coli* and *Campylobacter jejuni* Strains Isolated from Retail Chicken Liver. Genome Announc 5:. https://doi.org/10.1128/genomeA.01350-17

54. Marasini D, Karki AB, Buchheim MA, Fakhr MK (2018) Phylogenetic Relatedness Among Plasmids Harbored by *Campylobacter jejuni* and *Campylobacter coli* Isolated From Retail Meats. Front Microbiol 9:2167. https://doi.org/10.3389/fmicb.2018.02167

55. Holo H, Nilssen O, Nes IF (1991) Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. cremoris: isolation and characterization of the protein and its gene. J Bacteriol 173:3879–3887. https://doi.org/10.1128/jb.173.12.3879-3887.1991

56. Kassaa IA, Rafei R, Moukhtar M, et al (2019) LABiocin database: a new database designed specifically for Lactic Acid Bacteria bacteriocins. Int J Antimicrob Agents. https://doi.org/10.1016/j.ijantimicag.2019.07.012

57. Langille MGI, Hsiao WWL, Brinkman FSL (2010) Detecting genomic islands using bioinformatics approaches. Nat Rev Microbiol 8:373–382. https://doi.org/10.1038/nrmicro2350

58. Wang Y, Zhang M, Deng F, et al (2014) Emergence of multidrug-resistant *Campylobacter* species isolates with a horizontally acquired rRNA methylase. Antimicrob Agents Chemother 58:5405–5412. https://doi.org/10.1128/AAC.03039-14

59. Qin S, Wang Y, Zhang Q, et al (2012) Identification of a novel genomic island conferring resistance to multiple aminoglycoside antibiotics in *Campylobacter coli*. Antimicrob Agents Chemother 56:5332–5339. https://doi.org/10.1128/AAC.00809-12

60. Marasini D, Fakhr MK (2016) Whole-Genome Sequencing of a *Campylobacter jejuni* Strain Isolated from Retail Chicken Meat Reveals the Presence of a Megaplasmid with Mu-

Like Prophage and Multidrug Resistance Genes. Genome Announc 4:. https://doi.org/10.1128/genomeA.00460-16

61. Chen Y, Mukherjee S, Hoffmann M, et al (2013) Whole-genome sequencing of gentamicin-resistant *Campylobacter coli* isolated from U.S. retail meats reveals novel plasmid-mediated aminoglycoside resistance genes. Antimicrob Agents Chemother 57:5398–5405. https://doi.org/10.1128/AAC.00669-13

62. Bang DD, Borck B, Nielsen EM, et al (2004) Detection of seven virulence and toxin genes of *Campylobacter jejuni* isolates from Danish turkeys by PCR and cytolethal distending toxin production of the isolates. J Food Prot 67:2171–2177

63. Fox JG, Rogers AB, Whary MT, et al (2004) Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Camplyobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infect Immun 72:1116–1125. https://doi.org/10.1128/iai.72.2.1116-1125.2004

64. Hickey TE, McVeigh AL, Scott DA, et al (2000) *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun 68:6535–6541. https://doi.org/10.1128/iai.68.12.6535-6541.2000

65. Faïs T, Delmas J, Serres A, et al (2016) Impact of CDT Toxin on Human Diseases. Toxins 8:. https://doi.org/10.3390/toxins8070220

66. Sen K, Lu J, Mukherjee P, et al (2018) *Campylobacter jejuni* Colonization in the Crow Gut Involves Many Deletions within the Cytolethal Distending Toxin Gene Cluster. Appl Environ Microbiol 84:. https://doi.org/10.1128/AEM.01893-17

67. Snipen L, Wassenaar TM, Altermann E, et al (2012) Analysis of evolutionary patterns of genes in *Campylobacter jejuni* and *C. coli.* Microb Inform Exp 2:8. https://doi.org/10.1186/2042-5783-2-8

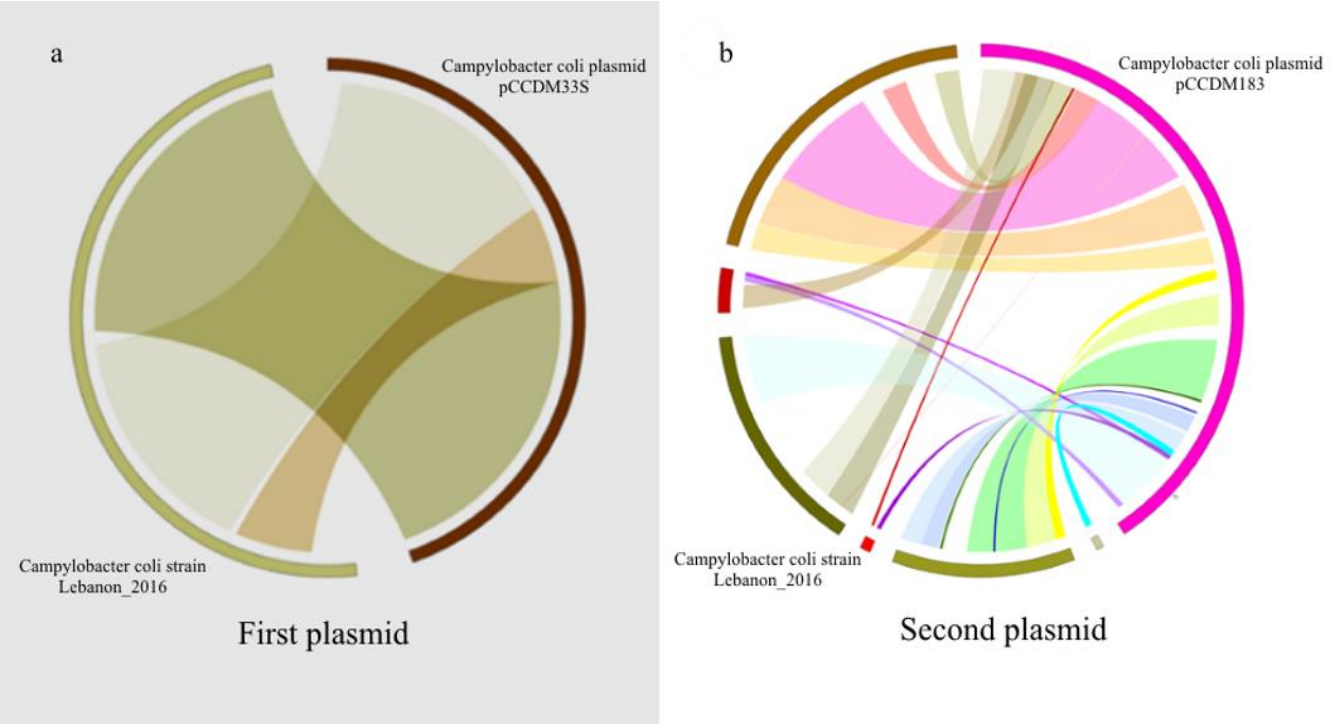
68. Dingle KE, Colles FM, Falush D, Maiden MCJ (2005) Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. J Clin Microbiol 43:340–347. https://doi.org/10.1128/JCM.43.1.340-347.2005

69. Nohra A, Grinberg A, Midwinter AC, et al (2016) Molecular Epidemiology of *Campylobacter coli* Strains Isolated from Different Sources in New Zealand between 2005 and 2014. Appl Environ Microbiol 82:4363–4370. https://doi.org/10.1128/AEM.00934-16

70. Fullerton KE, Ingram LA, Jones TF, et al (2007) Sporadic *campylobacter* infection in infants: a population-based surveillance case-control study. Pediatr Infect Dis J 26:19–24. https://doi.org/10.1097/01.inf.0000247137.43495.34

71. Friedman CR, Hoekstra RM, Samuel M, et al (2004) Risk factors for sporadic

Campylobacter infection in the United States: A case-control study in FoodNet sites. Clin Infect Dis Off Publ Infect Dis Soc Am 38 Suppl 3:S285-296. https://doi.org/10.1086/381598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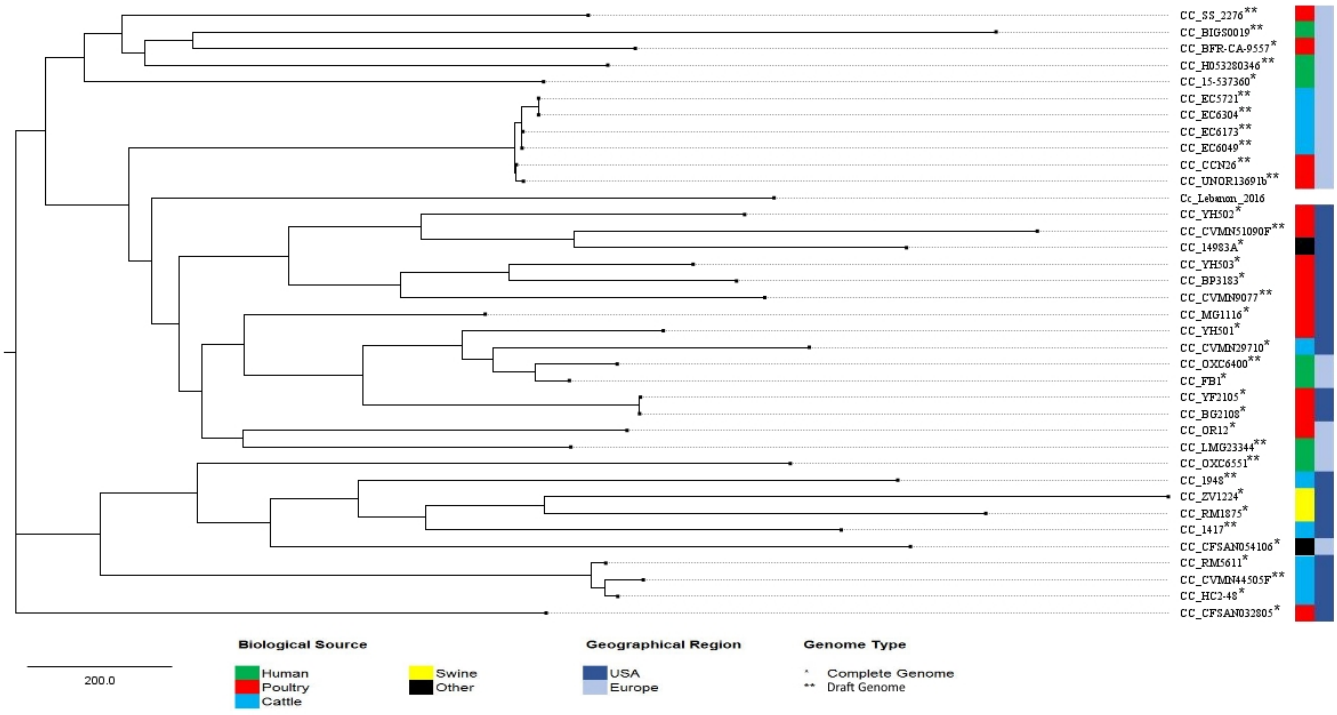
Campylobacter coli strain Lebanon_2016
First plasmid
b
Campylobacter coli plasmid pCCDM183
Campylobacter coli strain Lebanon_2016
Second plasmid

Fig 1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CC_EC6173**
CC_EC6049**
CC_CCN26**
CC_UNOR13691b**
Cc_Lebanon_2016
CC_YH502*
CC_CVMN51090F**
CC_14983A*
CC_YH503*
CC_BP3183*
CC_CVMN9077**
CC_MG1116*
CC_YH501*
CC_CVMN29710*
CC_OXC6400**
CC_FB1*
CC_YF2105*
CC_BG2108*
CC_OR12*
CC_LMG23344**
CC_OXC6551**
CC_1948**
CC_ZV1224*
CC_RM1875*
CC_1417**
CC_CFSAN054106*
CC_RM5611*
CC_CVMN44505F**
CC_HC2-48*
CC_CFSAN032805*
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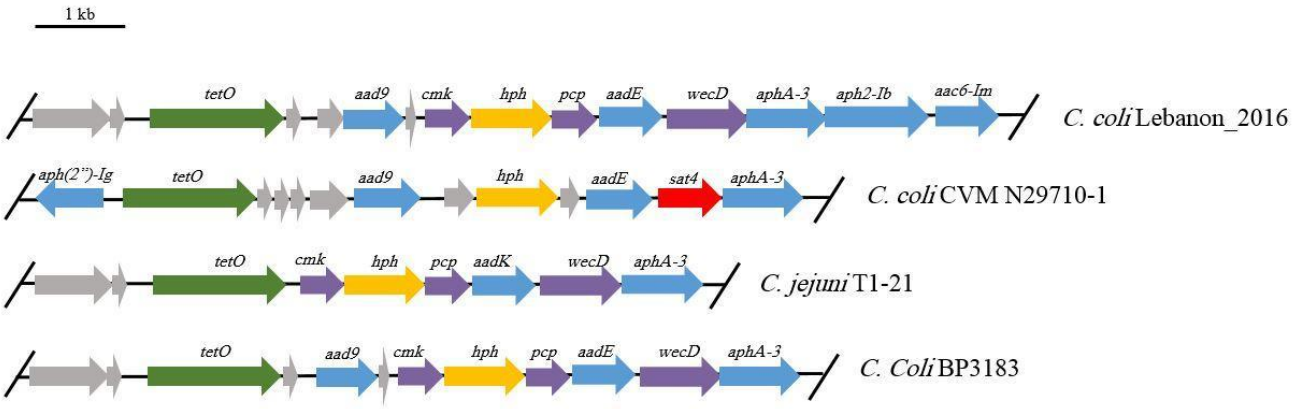
Poultry
Cattle
Swine
Other
Geographical Region
USA
Europe
Genome Type
* Complete Genome
** Draft Genome

Fig 2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aph(2")-Ig
tetO
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hph
aadE
sat4
aphA-3
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tetO
cmk
hph
pcp
aadK
wecD
aphA-3
C. jejuni T1-21
tetO
aad9
cmk
hph
pcp
aadE
wecD
aphA-3
C. Coli BP3183

Fig 3

Annex and Supplementary data:

Annex 1

Table 1: Biological and geographical sources of NCBI referenced complete and draft *C. coli* genomes.

NCBI referenced strain	Strain's code used in this study	Source	Country	Accession Number	
Campylobacter coli 84-2	Cc. 84-2	Swine	USA	NZ_AIMS01000004.1	Draft genomes
Campylobacter coli 1417	Cc. 1417	Bovine	USA	NZ_AIMY00000000.1	
Campylobacter coli 1948	Cc. 1948	Bovine	USA	NZ_AINE00000000.1	
Campylobacter coli BIGS0019	Cc. BIGS0019	Human	UK	NZ_ANHD00000000.1	
Campylobacter coli LMG23344	Cc. LMG23344	Human	Belgium	NZ_AINP00000000.1	
Campylobacter coli OXC6551	Cc. OXC6551	Human	UK	NZ_CUVK00000000.1	
Campylobacter coli OXC6400	Cc. OXC6400	Human	UK	NZ_CUNC00000000.1	
Campylobacter coli CVMN51090F	Cc. CVMN51090F	Turkey	UK	NZ_LBDO00000000.1	
Campylobacter coli CVMN9077	Cc. CVMN9077	Poultry	USA	NZ_JOVF00000000.1	
Campylobacter coli CVM41955	Cc. CVM41955	Human	USA	NZ_JAJP00000000.1	
Campylobacter coli EC6049	Cc. EC6049	Cattle	USA	NZ_FBFE01000000.1	
Campylobacter coli CCN26	Cc. CCN26	Poultry	UK	NZ_FBMP01000000.1	
Campylobacter coli SS_2276	Cc. SS_2276	Poultry	UK	NZ_FBEX01000000.1	
Campylobacter coli EC5721	Cc. EC5721	Cattle	UK	NZ_FBFG01000000.1	
Campylobacter coli EC6173	Cc. EC6173	Cattle	UK	NZ_FBCV01000000.1	
Campylobacter coli H053280346	Cc. H053280346	Human	UK	NZ_FBOY01000000.1	
Campylobacter coli CCN398	Cc. CCN398	Poultry	UK	NZ_FBMR01000000.1	
Campylobacter coli CVMN23392	Cc. CVMN23392	Poultry	USA	NZ_JOUM00000000.1	
Campylobacter coli UNOR13691b	Cc. UNOR13691b	Poultry	UK	NZ_FBMF01000000.1	
Campylobacter coli EC6304	Cc. EC6304	Cattle	UK	NZ_FBCC01000000.1	
Campylobacter coli RM5611	Cc. RM5611	Bovine	USA	NZ_CP007179.1	Complete genome
Campylobacter coli FB1	Cc. FB1	Human	UK	NZ_CP011015.1	
Campylobacter coli HC2-48	Cc. HC2-48	Cattle	USA	NZ_CP013034.1	
Campylobacter coli YH501	Cc. YH501	Poultry	USA	NZ_CP015528.1	
Campylobacter coli YH502	Cc. YH502	Poultry	USA	NZ_CP018900.1	
Campylobacter coli YH503	Cc. YH503	Poultry	USA	NZ_CP025281.1	
Campylobacter coli YF2105	Cc. YF2015	Poultry	USA	NZ_CP017865.1	

Campylobacter coli CFSAN032805	Cc. CFSAN032805	Poultry	USA	NZ_CP023545.1	
Campylobacter coli BG2108	Cc. BG2108	Poultry	USA	NZ_CP017878.1	
Campylobacter coli CVMN29710	Cc. CVMN29710	Cattle	USA	NC_022347.1	
Campylobacter coli BP3183	Cc. BP3183	Poultry	USA	NZ_CP017871.1	
Campylobacter coli 14983A	Cc. 14983A	Housefly	USA	NZ_CP017025.1	
Campylobacter coli RM1875	Cc. RM1875	Swine	USA	NZ_CP007183.1	
Campylobacter coli ZV1224	Cc. ZV1224	Swine	USA	NZ_CP017875.1	
Campylobacter coli CFSAN054106	Cc. CFSAN054106	Food	Denmark	NZ_CP028187.1	
Campylobacter coli OR12	Cc. OR12	Poultry	UK	NZ_CP013733.1	
Campylobacter coli MG116	Cc. MG116	Poultry	USA	NZ_CP017868.1	
Campylobacter coli BFR-CA-9557	Cc. BFR-CA-9557	Poultry	Germany	CP011777.1	
Campylobacter coli 15-537360	Cc. 15-537360	Human	UK	NC_022660.1	

Annex 2

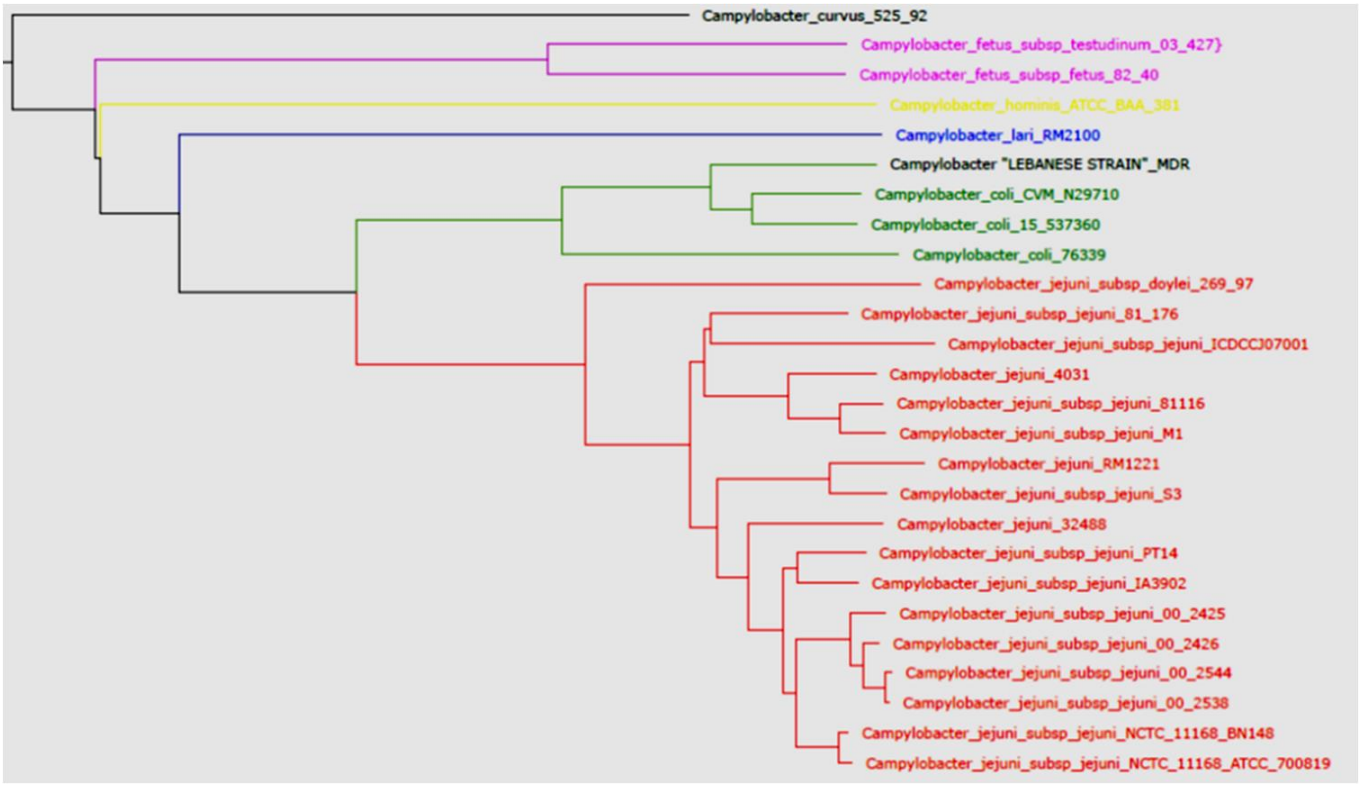

Figure 1: CVTree phylogenetic tree based on the nucleic sequences of *C. coli* Lebanon_2016 in Black, and other CVTree database *Campylobacter* genomes.

Chapter III: Investigation of XDR-*Acinetobacter baumannii* ST2 outbreak in an intensive care unit of a Lebanese tertiary care hospital

Introduction:

Acinetobacter baumannii is one of the main opportunistic pathogens known to cause Healthcare-Associated Infections (HAIs) and to develop a high-level of AMR [1]. Notably, its outstanding ability to cause real outbreaks similar to that of meticillin-resistant *Staphylococcus aureus* led some authors to qualify *A. baumannii* as gram-negative MRSA [2]. Recently, the WHO listed the carbapenem resistant *A. baumannii* (CRAB) within the pathogens of critical priority for research and development of novel antibiotics [3]. Meanwhile, the HAIs caused by multi-drug resistant (MDR) and extremely-drug resistant (XDR) *A. baumannii* strains are increasingly reported worldwide, especially in the Mediterranean region [4, 5]. This epidemic behavior motivated many researchers and epidemiologists throughout the world to develop typing methods [6], in order to delineate phylogenetic relationships between isolates, define circulating clones, and control outbreaks.

In this chapter, we aimed 1) to investigate the epidemiological links between CRAB strains isolated between January 2016 and July 2017 from ICU in a tertiary care hospital El Youssef Hospital Center (YHC) in North Lebanon in order to rule in or out the occurrence of an outbreak and elucidate its nature, 2) to describe the implemented strategy to eradicate epidemic clones and prevent the emergence of new CRAB infections.

The study involves 20 *A. baumannii* isolates recovered from YHC during two episodes (13 isolates from January to September 2016 and 7 isolates from April and July 2017), as well as 8 control isolates from Tripoli Gouvremental Hospital (TGH) and El NiNi Hospital (NH) recovered from the same period. The AMR testing hinted for the presence of two outbreak episodes where the XDR and MDR strains caused respectively the first and the second episodes. MLST analysis according to the Pasteur scheme (https://pubmlst.org/bigsdb?db=pubmlst_abaumannii_pasteur_seqdef) defined most (26/28) of isolates as belonging to ST2, and only two isolates belonged to ST415 (a member of clonal complex 2, differing only by a single locus from ST2), and ST578. Molecular typing using PFGE was superior to both antibiotyping and MLST and unveiled the polyclonal nature of the outbreak by detecting the presence of five clusters (I to V) and five unique profiles. The first episode was caused principally by the largest cluster II/ST2 but the second was completely induced by other PFGE types essentially sporadic or small clusters of two isolates. Moreover, the molecular characterization of AMR determinants demonstrated the predominance of *bla*$_{\text{OXA-23}}$ as a carbapenemase-encoding gene. Our results fit well with other Lebanese and worldwide studies demonstrating a shift toward international clone II with *bla*$_{\text{OX-23}}$ being the main carbapenem resistance mechanism [7–10]. Besides the typing approach, the eradication

of the appearance of new cases of CRAB has been achieved by the application of appropriate hygiene measures and the reduction of the empirical use of antibiotics.

Although, WGS-based typing approaches are increasingly used over the world particularly in developed countries, the traditional molecular typing methods PFGE and MLST are still valuable methods, and considered until recently gold standard methods for small and large scales of epidemiological investigations respectively [6]. This study highlights the added benefit of traditional molecular typing in elucidating the polyclonal nature of the outbreak and delineating the clonal relationships between isolates. Antimicrobial stewardship programs along with hygiene measures are other complementary steps that can act synergistically together with molecular typing in controlling *A. baumannii* outbreak.

REFERENCES

1. Haque M, Sartelli M, McKimm J, Abu Bakar M (2018) Health care-associated infections - an overview. Infect Drug Resist 11:2321–2333. https://doi.org/10.2147/IDR.S177247

2. Joly-Guillou M-L (2005) Clinical impact and pathogenicity of *Acinetobacter*. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 11:868–873. https://doi.org/10.1111/j.1469-0691.2005.01227.x

3. WHO (2017) WHO publishes list of bacteria for which new antibiotics are urgently needed. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

4. Kempf M, Rolain J-M, Azza S, et al (2013) Investigation of *Acinetobacter baumannii* resistance to carbapenems in Marseille hospitals, south of France: a transition from an epidemic to an endemic situation. APMIS Acta Pathol Microbiol Immunol Scand 121:64–71. https://doi.org/10.1111/j.1600-0463.2012.02935.x

5. Benmahmod AB, Said HS, Ibrahim RH (2019) Prevalence and Mechanisms of Carbapenem Resistance Among *Acinetobacter baumannii* Clinical Isolates in Egypt. Microb Drug Resist Larchmt N 25:480–488. https://doi.org/10.1089/mdr.2018.0141

6. Rafei R, Osman M, Dabboussi F, Hamze M (2019) Update on the epidemiological typing methods for *Acinetobacter baumannii*. Future Microbiol 14:1065–1080. https://doi.org/10.2217/fmb-2019-0134

7. Rafei R, Dabboussi F, Hamze M, et al (2014) Molecular analysis of *Acinetobacter baumannii* strains isolated in Lebanon using four different typing methods. PloS One

9:e115969. https://doi.org/10.1371/journal.pone.0115969

8. Nawfal Dagher T, Al-Bayssari C, Chabou S, et al (2019) Investigation of multidrug-resistant ST2 *Acinetobacter baumannii* isolated from Saint George hospital in Lebanon. BMC Microbiol 19:29. https://doi.org/10.1186/s12866-019-1401-2

9. Abhari SS, Badmasti F, Modiri L, et al (2019) Circulation of imipenem-resistant *Acinetobacter baumannii* ST10, ST2 and ST3 in a university teaching hospital from Tehran, Iran. J Med Microbiol 68:860–865. https://doi.org/10.1099/jmm.0.000987

10. Mathlouthi N, Ben Lamine Y, Somai R, et al (2018) Incidence of OXA-23 and OXA-58 Carbapenemases Coexpressed in Clinical Isolates of *Acinetobacter baumannii* in Tunisia. Microb Drug Resist Larchmt N 24:136–141. https://doi.org/10.1089/mdr.2016.0306

Article

Investigation of XDR-*Acinetobacter baumannii* ST2 outbreak in an intensive care unit of a Lebanese tertiary care hospital

Marwan Osman[1,‡], Fatima Bachir Halimeh[1,2,‡], Rayane Rafei[1], Hassan Mallat[1,3], Jad El Tom[3], Elie Bou Raad[3], Seydina M. Diene[2], Sabah Jamal[1], Ahmad Al Atrouni[1], Fouad Dabboussi[1], Walid Moudani[1], Jean-Yves Madec[4], Estelle Saras[4], Agnese Lupo[4], Marisa Haenni[4], Jean-Marc Rolain[2], Monzer Hamze[1,*]

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[1] Laboratoire Microbiologie Santé et Environnement (LMSE), Doctoral School of Science and Technology, Faculty of Public Health, Lebanese University, Tripoli, Lebanon.

[2] Aix-Marseille University, IRD, APHM, MEPHI, IHU Méditerranée infection, Faculté de Médecine et de Pharmacie, 19-21 boulevard Jean Moulin, 13385 Marseille CEDEX 05, France.

[3] El Youssef Hospital Center, Halba, Lebanon

[4] Université de Lyon – ANSES laboratoire de Lyon, Unité Antibiorésistance et Virulence Bactériennes, France.

* Corresponding author

Monzer Hamze: mhamze@monzerhamze.com

Running head:

Investigation of CRAB in a Lebanese hospital

Abstract

Aims: We sought to investigate the genetic epidemiological relatedness of healthcare-associated carbapenem-resistant *Acinetobacter baumannii* (CRAB) infections in a Lebanese tertiary care hospital.

Methods: Twenty-eight non-duplicate CRAB isolates detected among hospitalized patients between January-2016 and July-2017 were studied by conventional techniques, as well as real-time PCR, PFGE, and MLST analyses.

Results: Twenty-seven isolates harbored *bla*$_{\text{OXA-23}}$, and one strain harbored simultaneously *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-23}}$. Molecular tools determined different causative PFGE clusters divided into two temporal episodes. II/ST2 caused principally the first outbreak episode (January-September 2016), but is completely replaced by other PFGE types essentially sporadic or small clusters of two isolates during the second episode (April-July 2017).

Conclusions: Our findings reported successful control of a polyclonal outbreak of OXA-23-producing ST2 CRAB.

Keywords:

Carbapenem-Resistant *Acinetobacter baumannii*; Hospital outbreak; Intensive care unit; Molecular typing; Epidemiology; Lebanon

The burden of healthcare-associated infections (HAIs) remains unclear in Lebanese care centers. This serious issue is mainly associated with higher mortality rates, prolonged hospital stays, excess costs, increased antimicrobial resistance (AMR), and other unfortunate consequences [1]. *Acinetobacter baumannii* is one of the main opportunistic Gram-negative pathogens known to cause serious HAIs and outbreaks. Considered as 'microbial weeds', these bacteria are ubiquitous and capable of causing a wide variety of HAIs including ventilator-associated pneumonia, surgical site and wound infections, and urinary tract and bloodstream infections, especially in intensive care units (ICUs) [2]. Regrettably, the incidence of HAIs caused by multidrug and extremely-drug resistant (MDR and XDR) *A. baumannii* isolates such as carbapenem-resistant *A. baumannii* (CRAB) is constantly increasing worldwide. Carbapenems have been the first appropriate choice for the treatment of severe *A. baumannii* infections, but the emergence of CRAB may significantly compromise their efficacy and reduce available treatment options. Nowadays, *A. baumannii* is a major challenge in public health in the Mediterranean region, with high levels of carbapenem resistance such as 98% in Egypt, 85% in Greece, 80% in Turkey, and 60% in Italy [3-6]. In Lebanon, several CRAB strains hosting β-lactamase producing genes were reported in clinical settings such as $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-143}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{GES-11}}$ [2]. According to a recent Lebanese investigation conducted in 13 tertiary care hospitals, 88% of *A. baumannii* isolates were resistant to carbapenems [7]. Besides the propensity to develop MDR, *A. baumannii* has an outstanding epidemic behavior. Increasingly observed worldwide outbreaks are generally caused by MDR strains belonging to a limited number of clones that successfully spread among tertiary care hospitals of different cities and even countries [8]. Here, we report the emergence of a large unexpected number of MDR and XDR *A. baumannii* infection cases in the ICU of El Youssef Hospital Center (YHC), Akkar. In this context, we investigated the genetic relatedness of MDR and XDR *A. baumannii* isolates in order to verify the eventual outbreak nature, to highlight epidemiological links among isolates, and to describe the successful strategy implemented to eradicate epidemic clones and avoid the emergence of new CRAB infections.

Material and methods

Study population

Twenty patients from YHC were enrolled in this study including 14 males and 6 females, ranging in age from 14 to 93 years, with a mean age of 56 ± 5.4 years. All patients were defined as suffering from HAIs according to the European Centre for Disease Prevention and Control definitions [9]. We retrieved data from the YHC's computerized ordering system and examined medical records of all ICU patients with *A. baumannii* infections admitted to the hospital between January 1, 2015–September 30, 2019. Clinical data including patient demographics, admission diagnosis, origin of infection, treatment regimen, and outcome were recorded. Information regarding carbapenem consumption and mortality rates were collected in the ICU of YHC during the same period.

Sampling, culture, and identification

A total of 20 non-repetitive *A. baumannii* strains were isolated from patients with HAIs admitted between January 2016 and July 2017 to the ICU of YHC, a 120-bed tertiary-care center in Halba, North Lebanon. All isolates were obtained at least after the first 3 days of hospital admission. To investigate if a regional outbreak was occurring, or if several clones were circulating, 8 *A. baumannii* strains were collected from patients in the ICU of Tripoli Governmental Hospital (TGH) (n = 7) and Nini Hospital (NH) (n = 1) located in the same geographical area during the same period of time. The strains were identified via a matrix-assisted laser adsorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) (Bruker, United States). All information about infected patients were recorded and summarized in Figure 1. All data were anonymized for subsequent analyses.

Antibiotic susceptibility testing

To determine the antibiotic susceptibility of the strains, the standard Kirby-Bauer disk diffusion method was performed on Mueller–Hinton agar as recommended by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) guidelines 2019. The antibiotics tested were ticarcillin, ticarcillin-clavulanate, piperacillin, piperacillin-tazobactam, ceftazidime, cefotaxime, cefepime, imipenem, meropenem, gentamicin, tobramycin, netilmicin, amikacin, fosfomycin, tetracycline, doxycycline, levofloxacin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Moreover, the minimal inhibitory concentration (MIC) of colistin was determined using the broth microdilution test according to EUCAST 2019. The phenotype of *A. baumannii* isolates is defined as MDR and XDR according to criteria published by Magiorakos *et al* [10].

Molecular characterization of β-lactamase encoding genes by real-time PCR

Bacterial DNA was extracted using the automatic robot EZ1 (Qiagen BioRobot EZ1, Tokyo, Japan), with the extraction kit (EZ1 DNA, Qiagen, Hilden, Germany), following the manufacturer's instructions. In order to screen the presence of carbapenemase-encoding genes, real-time PCR was performed using primers detecting *bla*$_{\text{NDM-1}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-48}}$, and *bla*$_{\text{OXA-58}}$ as described previously [11]. Positive and negative controls were used in each assay.

Pulsed-field gel electrophoresis (PFGE)

DNA of *A. baumannii* isolates was digested with *Apa*I (Promega, Madison, Wisconsin, USA) as a restriction enzyme, separated by electrophoresis on a 1% agarose gel (Bio-Rad) in 0.5X Tris/borate/ethylenediaminetetraacetic acid (TBE) buffer at 14 °C using a CHEF Maper XA system (Bio-Rad) with 6 V/cm, and pulse times ranging from 5 to 20 s for 19 h [12]. PFGE profiles were processed using the module Gelcompare (v. 6.6) of the Bionumerics software (Applied Maths, v. 7.1). A phylogenetic tree was constructed by UPGMA (unweighted pair group method with arithmetic mean) allowing 1% tolerance and 1% optimization.

Multilocus sequence typing (MLST)

The genetic relationship among isolates was determined by MLST according to the Pasteur scheme (https://pubmlst.org/abaumannii/) [13, 14]. The internal fragments of these genes were amplified, purified and sequenced using an ABI 3130XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequence types (STs) were analyzed using the *A. baumannii* MLST database.

Results

A total of 28 non-duplicate strains isolated from hospitalized patients in the ICUs of three major hospitals in North Lebanon were identified by MALDI-TOF-MS as *A. baumannii*. A first group of 20 isolates was collected in YHC from January to September 2016 (episode 1 = 13 isolates) and from April to July 2017 (episode 2 = 7 isolates). Isolates from the first episode were mostly XDR *A. baumannii* isolates, while isolates from the second episode were mostly characterized as MDR. The majority of cases were considered as ventilator-associated pneumonia (85%). A second group consisted of 8 XDR *A. baumannii* isolates recovered from two other hospitals in North Lebanon.

Antibiotic susceptibility testing results revealed that all isolates were resistant to β-lactams including carbapenems. In addition, 95% of the isolates were resistant to fluoroquinolones, 85% to tobramycin, 70% to amikacin, 80% to doxycycline, and 60% to

trimethoprim/sulfamethoxazole. None of the isolates was resistant to colistin (MIC < 2 μg/ml). The *bla*$_{\text{OXA-23}}$ gene was detected in 96.4% (27/28) of the isolates, while one isolate was negative for all the tested carbapenemase-encoding genes. One *bla*$_{\text{OXA-23}}$-carrying isolate simultaneously displayed the *bla*$_{\text{NDM-1}}$ gene.

MLST analysis showed that 92.9% (26/28) of the *A. baumannii* isolates belonged to ST2, whereas two isolates were assigned to ST415 (single locus variant (SLV) of ST2) and ST578. ST2 was identified in YHC (18/20), TGH (7/7), and Nini Hospital (1/1) (Figure 1). The two other STs were only detected in YHC.

PFGE profiles revealed the presence of five clusters and five unique profiles when adopting a Dice similarity index >90% as a cut-off for delineation clusters. Most of the YHC isolates grouped into four clusters: cluster I, II, IV, and V. The cluster II was the largest one encompassing 12 XDR *A. baumannii* isolates recovered from the YHC (11 isolates) and TGH (one isolate). The cluster IV grouped only two XDR isolates obtained in 2016 from the YHC and the TGH hospitals. The cluster IV's isolates belonged to clonal complex 2 (CC2) with ST2 and ST415. Each of the clusters I and V included two isolates with the MDR phenotype obtained from the YHC hospital in 2017. Of note, the cluster I contained the isolate co-harboring the *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-23}}$ carbapenemases. Additionally, 3 sporadic isolates with both MDR and XDR phenotypes isolated in 2017 at YHC yielded distinct PFGE profiles, of which one belonged to ST578. Regarding the 8 isolates, 6 displayed different pulostypes from the YHC isolates where they gave one cluster (cluster III) and two singletons. The cluster (III) contained four isolates from TGH and NH hospitals isolated in 2016.

Control and elimination of MDR- and XDR-*Acinetobacter baumannii* cases

The infection control committee (ICC) at YHC set up an antimicrobial stewardship and infection prevention and control programs. After the emergence of the first *A. baumannii* cases, several measures were carried out in the ICU. The ICC created an outbreak management team that has executed an educational program on infection control, isolation, and precautions for all healthcare workers in the ICU. In addition, a new *A. baumannii* carriage screening policy has been implemented for ICU patients at admission and weekly thereafter. Sputum samples were also collected for culture every fifth day for intubated patients with abundant secretions. In the case of the emergence of a new CRAB infection, the patient was treated using a colistin based treatment and cared for strict isolation precautions. Moreover, the team promoted and

ensured compliance with standard hygiene measures in the ICU, and also with intensified cleaning and disinfecting of materials, equipment, facilities, premises, vehicles, and surfaces present in the department. Hydrogen peroxide was used for routine cleaning and environmental decontamination in ICU rooms. After patient discharge, conventional routine terminal cleaning was followed by hydrogen peroxide vapor disinfection treatment. In January 2018, the ICC approved a plan to restrict the use of empiric carbapenem drugs for ICU patients as part of a stewardship program. The prudent use of carbapenems did not correlate with an increased mortality rate in the ICU of YHC but clearly decreased the proportion of MDR isolates identified in this ward, including XDR *A. baumannii* (Figure 2).

Discussion

A. baumannii has been described as one of the most successful Gram-negative bacteria responsible for HAIs and outbreaks, particularly in immunocompromised patients admitted to ICUs [15]. A large unexpected number of CRAB was observed in YHC, between January 2016 to July 2017. However, two different temporal episodes were observed with a crude death rate of 31.6% (6/19). Unfortunately, multi-resistant pathogens, including CRAB, has become a significant health problem in Lebanon as well as globally in the 21st century [2, 16-18].

In order to track these cases and to control the spread of CRAB in the hospital, several typing methods depending on phenotypic and genotypic tools were used. Antibiotic susceptibility testing orientated us to the presence of two different outbreaks, indicating slight differences between the profiles of isolates during temporal episodes 1 and 2. PFGE results confirmed that the first outbreak episode is due primarily to a large XDR *A. baumannii* clone (cluster II) while the second outbreak episode is caused essentially by two small MDR clones (each formed by 2 isolates) and three XDR and MDR sporadic isolates. In fact, the cluster II was the largest one encompassing 12 isolates where 11 predominately with XDR phenotype originated from patients of the YHC ICU during 2016 suggesting the occurrence of an outbreak, and one with XDR phenotype collected from TGH in 2016 supposing a more limited expansion for this clone outside the YHC. Notably, we have noted the clustering of 2 TGH isolates within the YHC clusters, as well as the clustering of isolates from TGH and NH hospitals in a small PFGE cluster (cluster III). These observations can be explained either by the circulation of isolates between hospitals or by the presence of homogenous CC2 clusters in Lebanon requiring more discriminative typing methods (such as whole-genome sequencing) to

unravel the true degree of their genetic relatedness. Similarly, Rafei *et al.* [19] have noted a great homogeneity within the ST2 clone wherein they found a single Lebanese national clone PFGE type A/ST2 circulated at many hospitals in both Tripoli and Beirut cities in 2011/2012. In this context, a more thorough analysis must be done in the future to dissect the extent of the spread of such homogenous clones in Lebanon.

Based on MLST results, our report showed that the majority of YHC isolates belonged to the ST2 (18/20; 90%). Similarly, all the isolates from the two other hospitals in the same geographical area belonged to the same ST. The ST2 clone, which constitutes the main ST in the CC2 or so-called international clone 2, has also been described as being the most common ST in numerous Mediterranean basin countries including Turkey, Italy, Greece, and Algeria [20, 21]. In fact, even if different ST clones have been described in Lebanon, the ST2 clone appears to be the main ST found over recent years. ST2 was widely distributed in clinical settings across the Lebanese governorates, accounting for around 60-90% of isolates [2]. Moreover, ST2 has been repeatedly associated with carbapenemase-producing isolates and hospital outbreaks worldwide [20, 22, 23]. On the other hand, two other STs, ST415 and ST578, were identified in YHC where ST415 belonged to the same CC of ST2 (CC2), but ST578 did not share any allele with ST2. These rare STs are reported here for the first time in Lebanon [2], while sporadic cases ST415 and ST578 have previously been reported among animal isolates in France [24] and a clinical isolate in Japan [25].

In our study, even the nature of the outbreak is polyclonal, the $bla_{\text{OXA-23}}$ gene was found in the majority of CRAB ST2 isolates (95%). OXA-23 carbapenemase is now recognized as the major source of carbapenem resistance in *A. baumannii* worldwide, including Lebanese hospitals [2]. Several *A. baumannii* outbreaks were reported in Lebanon where a shift from OXA-58 to OXA-23 was witnessed [2]. An example but not limited to, a recent investigation of a polyclonal CRAB outbreak at a large Lebanese tertiary hospital carried out at the same time of this study also showed the predominance of OXA-23 [26]. Moreover, one strain was simultaneously positive for the $bla_{\text{OXA-23}}$ and $bla_{\text{NDM-1}}$ genes. Previous cases of CRAB carrying the $bla_{\text{NDM-1}}$ gene were reported among Syrian (ST85) and Lebanese patients (ST25) [8]. Al Atrouni *et al.* also reported the first detection of *Acinetobacter pittii* clinical isolates carrying the $bla_{\text{NDM-1}}$ gene in Lebanon [27]. The aforementioned data revealed the trend of the spread of the $bla_{\text{NDM-1}}$ gene in *Acinetobacter* spp. in Lebanon, highlighting thus the clinical relevance of these dangerous resistant bacteria.

The prudent use of carbapenems in YHC had a dramatic effect on the emergence of

MDR bacterial isolates. Our findings are in accordance with a recent study, which showed a decrease in the level of carbapenem consumption was accompanied by a rise in the susceptibility of *A. baumannii* and a reduction in the emergence *bla*$_{\text{OXA-23}}$ producing CRAB ST2 [28]. The aforementioned measures helped to decrease the number of *A. baumannii* infection cases, leading to a full eradication from August 2017 to November 2019.

Conclusion

This study reported successful control of a polyclonal outbreak of OXA-23-producing CRAB ST2 in patients admitted to the ICU of a tertiary care hospital in Lebanon. To our knowledge, even though ST2 has become endemic in Lebanese clinical settings, ST415 and ST578 were described for the first time in Lebanon. HAIs can occur in any healthcare setting, but the compliance with the infection control strategy and policies adopted in YHC has enhanced the prevention of the development of new cases, and thus, the eradication of XDR *A. baumannii* ST2 from the ICU department.

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Summary Points:

- Emergence of carbapenem-resistant *Acinetobacter baumannii* outbreak in an intensive care unit of a Lebanese tertiary care hospital between January 2016 to July 2017.
- Patients with CRAB infection enrolled in this study were defined as suffering from healthcare associated infections according to the European Centre for Disease Prevention and Control definitions.
- Antibiotic susceptibility testing determined the presence of two different episodes, showing slight differences between the resistance patterns of isolates during temporal episodes 1 (January-September 2016) and 2 (April-July 2017).
- Molecular characterization of β-lactamase genes and Multilocus sequence typing findings indicated that OXA-23-producing ST2 *A. baumannii* is the main circulating clone.
- Pulsed-field gel electrophoresis results confirmed that the first outbreak episode is due primarily to a large extensively-drug-resistant (XDR) *A. baumannii* clone (cluster II) while the second outbreak episode is caused essentially by two small multidrug-resistant (MDR) clones, and three XDR and MDR sporadic isolates.
- II/ST2 caused principally the first outbreak episode, but is completely replaced by other PFGE types essentially sporadic or small clusters of two isolates during the second episode.
- Two rare subtypes, ST415 and ST578, were described for the first time in Lebanon.
- Multifaceted approaches in infection control allowed the eradication of epidemic clones, and avoided the spread of new CRAB infections.

Figure 1. Features of carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolates detected in El Youssef Hospital Center in Lebanon during January 2016- July 2017. The dendrogram was generated by UPGMA (unweighted pair group method with arithmetic mean) using Gelcompare (v. 6.6) on *ApaI* restriction profiles. The clusters delineated at >90% similarity cut-off were limited by rectangles. Note: XDR: extensive drug resistant; MDR: multidrug resistant; CST: colistin; AMK: amikacin; TMN: tobramycin; LVX: levofloxacin; DOX: doxycycline; SXT: trimethoprim-sulfamethoxazole; NT: not typable.

Figure 2. Carbapenem-resistant *Acinetobacter baumannii* (CRAB) strains isolated in patients in the intensive care unit (ICU) versus carbapenem consumption in the same department of El Youssef Hospital Center, during January 1, 2015–September 30, 2019. ICU mortality rates were also shown in the same period. DOT, days of therapy; PD, patient days.

References

1. Haque M, Sartelli M, McKimm J, Abu Bakar M. Health care-associated infections - an overview. *Infect Drug Resist.* 11: 2321-33 (2018).

2. Jamal S, Al Atrouni A, Rafei R, Dabboussi F, Hamze M, Osman M. Molecular mechanisms of antimicrobial resistance in *Acinetobacter baumannii*, with a special focus on its epidemiology in Lebanon. *J Glob Antimicrob Resist.* 15: 154-63 (2018).

3. Kempf M, Rolain JM, Azza S, *et al.* Investigation of *Acinetobacter baumannii* resistance to carbapenems in Marseille hospitals, south of France: a transition from an epidemic to an endemic situation. *APMIS.* 121: 64-71 (2013).

4. Benmahmod AB, Said HS, Ibrahim RH. Prevalence and Mechanisms of Carbapenem Resistance Among *Acinetobacter baumannii* Clinical Isolates in Egypt. *Microb Drug Resist.* 25 (4), 480-488 (2018).

5. Lorenzin G, Scaltriti E, Gargiulo F, *et al.* Extensively drug-resistant *Acinetobacter baumannii* isolated from intensive care units in northern Italy: a genomic approach to characterize new sequence types. *Future Microbiol.* 14:1281-92 (2019).

6. Karampatakis T, Antachopoulos C, Tsakris A, Roilides E. Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* in Greece: an extended review (2000-2015). *Future Microbiol.*12:801-15 (2017).

7. Moghnieh R, Araj GF, Awad L, *et al.* A compilation of antimicrobial susceptibility data from a network of 13 Lebanese hospitals reflecting the national situation during 2015-2016. *Antimicrob Resist Infect Control.* 8: 41 (2019).

8. Al Atrouni A, Hamze M, Jisr T, *et al.* Wide spread of OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* belonging to clonal complex II in different hospitals in Lebanon. *Int J Infect Dis.* 52: 29-36 (2016).

9. Hopmans TEM, Smid EA, Wille JC, *et al.* Trends in prevalence of healthcare-associated infections and antimicrobial use in hospitals in the Netherlands: 10 years of national point-prevalence surveys. *J Hosp Infect.* 104: 181-7 (2020).

10. Magiorakos AP, Srinivasan A, Carey RB, *et al.* Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect.* 18: 268-81 (2012).

11. Mellouk FZ, Bakour S, Meradji S, *et al.* First Detection of VIM-4-Producing *Pseudomonas aeruginosa* and OXA-48-Producing *Klebsiella pneumoniae* in Northeastern (Annaba, Skikda) Algeria. *Microb Drug Resist.* 23: 335-44 (2017).

12. Seifert H, Dolzani L, Bressan R, *et al.* Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. *J Clin Microbiol.* 43: 4328-35 (2005).

13. Diancourt L, Passet V, Nemec A, Dijkshoorn L, Brisse S The population structure of *Acinetobacter baumannii*: expanding multiresistant clones from an ancestral susceptible genetic pool. *PLoS One*. 5: e10034 (2010).

14. Rafei R, Osman M, Dabboussi F, Hamze M Update on the epidemiological typing methods for *Acinetobacter baumannii*. *Future Microbiol.* 14: 1065-80 (2019).

15. Blanco N, Harris AD, Rock C, *et al.* Risk Factors and Outcomes Associated with Multidrug-Resistant *Acinetobacter baumannii* upon Intensive Care Unit Admission. *Antimicrob Agents Chemother*. 62 (1) (2018).

16. Al Omari S, Al Mir H, Wrayde S, *et al.* First Lebanese Antibiotic Awareness Week campaign: knowledge, attitudes and practices towards antibiotics. *J Hosp Infect.* 101: 475-9 (2019).

17. Al-Mir H, Osman M, Azar N, Madec JY, Hamze M, Haenni M. Emergence of clinical *mcr*-1-positive *Escherichia coli* in Lebanon. *J Glob Antimicrob Resist.* 19: 83-4 (2019).

18. Osman M, Al Mir H, Rafei R, *et al.* Epidemiology of antimicrobial resistance in Lebanese extra-hospital settings: An overview. *J Glob Antimicrob Resist.* 17: 123-9 (2019).

19. Rafei R, Dabboussi F, Hamze M, *et al.* Molecular analysis of *Acinetobacter baumannii* strains isolated in Lebanon using four different typing methods. *PLoS One*. 9: e115969 (2014).

20. Agodi A, Voulgari E, Barchitta M, *et al.* Spread of a carbapenem- and colistin-resistant *Acinetobacter baumannii* ST2 clonal strain causing outbreaks in two Sicilian hospitals. *J Hosp Infect.* 86: 260-6 (2014).

21. Bakour S, Olaitan AO, Ammari H, *et al.* Emergence of Colistin- and Carbapenem-Resistant *Acinetobacter baumannii* ST2 Clinical Isolate in Algeria: First Case Report. *Microb Drug Resist.* 21: 279-85 (2015).

22. Villalon P, Valdezate S, Medina-Pascual MJ, Rubio V, Vindel A, Saez-Nieto JA. Clonal diversity of nosocomial epidemic *Acinetobacter baumannii* strains isolated in Spain. *J Clin Microbiol.* 49: 875-82 (2011).

23. Kubo Y, Komatsu M, Tanimoto E, *et al.* Spread of OXA-23-producing *Acinetobacter baumannii* ST2 and ST246 in a hospital in Japan. *J Med Microbiol.* 64: 739-44 (2015).

24. Godeux AS, Lupo A, Haenni M, *et al.* Fluorescence-Based Detection of Natural Transformation in Drug-Resistant *Acinetobacter baumannii*. *J Bacteriol.* 200 (19) (2018).

25. Tada T, Miyoshi-Akiyama T, Shimada K, *et al.* Dissemination of clonal complex 2 *Acinetobacter baumannii* strains co-producing carbapenemases and 16S rRNA methylase ArmA in Vietnam. *BMC Infect Dis*. 15: 433 (2015).

26. Makke G, Bitar I, Salloum T, *et al.* Whole-Genome-Sequence-Based Characterization of Extensively Drug-Resistant *Acinetobacter baumannii* Hospital Outbreak. *mSphere*. 5 (1) (2020).

27. Al Atrouni A, Joly-Guillou ML, Hamze M, Kempf M. Emergence of NDM-1 and OXA-72 producing *Acinetobacter pittii* clinical isolates in Lebanon. *New Microbes New Infect.* 12: 43-4 (2016).

28. Chamieh A, Nawfal TD, Ballouz T, *et al.* Control and elimination of extensively drug-resistant *Acinetobacter baumanii* in an intensive care unit. *Emerg Infect Dis*. 25: 1928-31 (2019).

Reference annotations

Papers of special note have been highlighted as: *of interest ** of considerable interest

2. Jamal S, Al Atrouni A, Rafei R, Dabboussi F, Hamze M, Osman M. Molecular mechanisms of antimicrobial resistance in *Acinetobacter baumannii*, with a special focus on its epidemiology in Lebanon. *J Glob Antimicrob Resist*. 15: 154-63 (2018).

**** An interesting review that discusses the molecular epidemiology of *Acinetobacter baumannii* in Lebanon.**

5. Lorenzin G, Scaltriti E, Gargiulo F, *et al.* Extensively drug-resistant *Acinetobacter baumannii* isolated from intensive care units in northern Italy: a genomic approach to characterize new sequence types. *Future Microbiol.* 14:1281-92 (2019).

*** OXA-23 carbapenemase is reported as almost exclusive carbapenemase in *Acinetobacter baumannii* in intensive care units in Italy.**

7. Moghnieh R, Araj GF, Awad L, *et al.* A compilation of antimicrobial susceptibility data from a network of 13 Lebanese hospitals reflecting the national situation during 2015-2016. *Antimicrob Resist Infect Control*. 8: 41 (2019).

**** This nationwide study showed that resistance to carbapenems, often the most active drug against *Acinetobacter baumannii* infections, is becoming more common in Lebanese clinical settings.**

8. Al Atrouni A, Hamze M, Jisr T, *et al.* Wide spread of OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* belonging to clonal complex II in different hospitals in Lebanon. *Int J Infect Dis*. 52: 29-36 (2016).

**** Delivers evidence of the wide dissemination of OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* belonging to international clone II in Lebanese hospitals.**

14. Rafei R, Osman M, Dabboussi F, Hamze M Update on the epidemiological typing methods for *Acinetobacter baumannii*. *Future Microbiol.* 14: 1065-80 (2019).

*** A useful review on *Acinetobacter baumannii* typing methods.**

19. Rafei R, Dabboussi F, Hamze M, *et al*. Molecular analysis of *Acinetobacter baumannii* strains isolated in Lebanon using four different typing methods. *PLoS One*. 9: e115969 (2014).

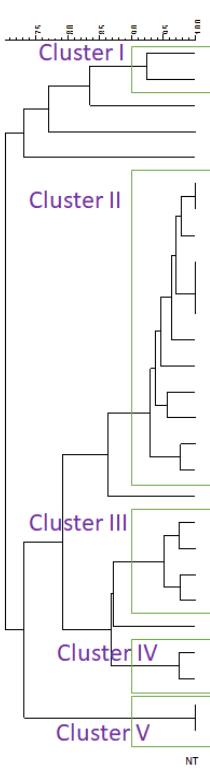
**** First detection of *bla*$_{\text{OXA-23}}$ in Lebanon.**

26. Makke G, Bitar I, Salloum T, *et al.* Whole-Genome-Sequence-Based Characterization of Extensively Drug-Resistant *Acinetobacter baumannii* Hospital Outbreak. *mSphere*. 5 (1) (2020).

**** First study investigating an extensively drug-resistant carbapenem resistant *Acinetobacter baumannii* outbreak using whole-genome-based approach in Lebanon.**

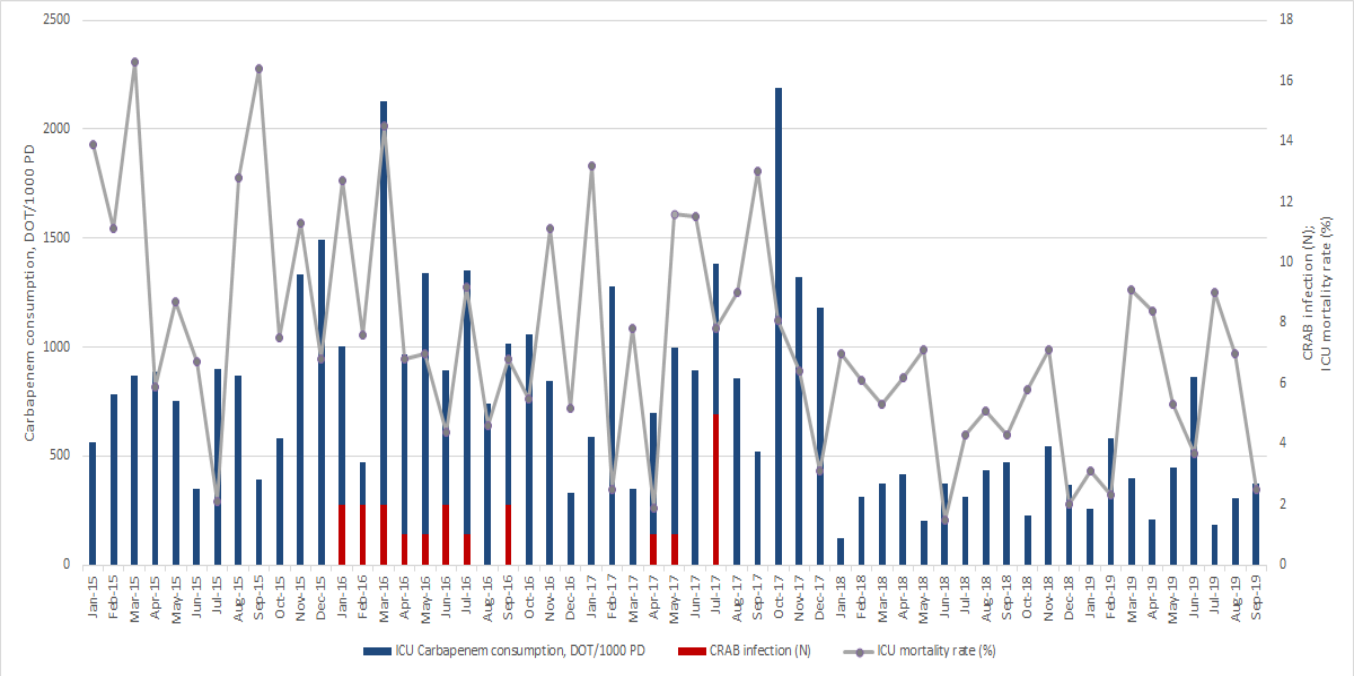
28. Chamieh A, Nawfal TD, Ballouz T, *et al.* Control and elimination of extensively drug-resistant *Acinetobacter baumanii* in an intensive care unit. *Emerg Infect Dis*. 25: 1928-31 (2019).

*** The decrease in the use of empiric carbapenems in the intensive care unit and the switch to colistin monotherapy for extensively drug-resistant *Acinetobacter baumannii* infections are associated with a high decrease in *bla*$_{\text{OXA-23}}$-carrying CRAB isolates in a Lebanese hospital.**



CMUL code	Hospital	Sex	Age of patients (years)	Cause of hospital admission	Outcome	Time delay to the detection of CRAB (days)	Origin	Date of isolation	Category	Susceptible antibiotic(s)	ST	Carbapenemase encoding gene
414	YHC	F	48	Respiratory symptoms	Recovery	14	Sputum	07/2017	MDR	AMK, DOX, SXT, CST	2	bla_{OXA23}
415	YHC	F	71	Stroke	Recovery	24	Blood	07/2017	MDR	TMN, AMK, DOX, SXT, CST	2	bla_{OXA23}, bla_{NDM1}
417	YHC	F	84	Septic shock	Death	7	Sputum	07/2017	XDR	SXT, CST	2	bla_{OXA23}
416	YHC	M	93	Respiratory symptoms	Recovery	6	Urine/Sputum	07/2017	XDR	SXT, CST	2	bla_{OXA23}
418	YHC	M	26	Road accident	Recovery	7	Sputum	07/2017	MDR	TMN, AMK, LVX, DOX, SXT, CST	578	-
398	YHC	M	28	Traumatic brain injury	Recovery	7	Sputum	09/2016	XDR	CST	2	bla_{OXA23}
400	YHC	M	14	Respiratory symptoms	Recovery	10	Sputum	09/2016	XDR	CST	2	bla_{OXA23}
378	YHC	M	77	Respiratory symptoms	Death	10	Sputum	03/2016	XDR	CST	2	bla_{OXA23}
368	YHC	M	27	Gunshot innjury	Recovery	16	Catheter	01/2016	MDR	AMK, SXT, CST	2	bla_{OXA23}
371	YHC	F	66	Respiratory symptoms	Unknown	8	Sputum	02/2016	XDR	CST	2	bla_{OXA23}
372	YHC	F	44	Respiratory symptoms	Recovery	3	Sputum	02/2016	XDR	CST	2	bla_{OXA23}
383	TGH	-	-	-	-	-	Wound	06/2016	XDR	SXT, CST	2	bla_{OXA23}
376	YHC	M	77	Respiratory symptoms	Death	3	Sputum	03/2016	XDR	CST	2	bla_{OXA23}
374	YHC	M	82	Respiratory symptoms	Death	3	Sputum	04/2016	XDR	CST	2	bla_{OXA23}
380	YHC	M	67	Gunshot injury	Recovery	5	Sputum	05/2016	XDR	CST	2	bla_{OXA23}
389	YHC	M	18	Gunshot injury	Recovery	12	Sputum	06/2016	XDR	CST	2	bla_{OXA23}
390	YHC	M	56	Road accident injury	Recovery	14	BAL	07/2016	XDR	CST	2	bla_{OXA23}
395	TGH	-	-	-	-	-	Wound	08/2016	XDR	AMK, CST	2	bla_{OXA23}
396	TGH	-	-	-	-	-	Wound	08/2016	XDR	CST	2	bla_{OXA23}
397	TGH	-	-	-	-	-	Catheter	08/2016	XDR	TMN, CST	2	bla_{OXA23}
391	TGH	-	-	-	-	-	Pleural fluid	07/2016	XDR	AMK, CST	2	bla_{OXA23}
393	NH	-	-	-	-	-	Rectal swab	06/2016	XDR	TMN, AMK, CST	2	bla_{OXA23}
392	TGH	-	-	-	-	-	Catheter	07/2016	XDR	CST	2	bla_{OXA23}
384	TGH	-	-	-	-	-	Wound	06/2016	XDR	SXT, CST	2	bla_{OXA23}
385	YHC	M	32	Septic shock	Death	8	Sputum	06/2016	XDR	CST	415	bla_{OXA23}
409	YHC	M	73	Stroke	Recovery	23	Wound	04/2017	MDR	TMN, AMK, DOX, SXT, CST	2	bla_{OXA23}
410	YHC	F	67	Stroke	Death	5	Sputum	05/2017	MDR	AMK, DOX, SXT, CST	2	bla_{OXA23}
367	YHC	M	70	Respiratory symptoms	Recovery	4	Sputum	01/2016	XDR	CST	2	bla_{OXA23}

Figure 2



Conclusion and Perspectives

For many years, the routine tasks of clinical microbiological laboratories in developing countries as Lebanon relied on expressed phenotypic traits of pathogens whatever for the traditional identification, AMR detection, and basic typing. Certainly, the applications of some advanced molecular techniques are limited to research laboratories and a few numbers of clinical laboratories. Research laboratories are also considered to be restricted in number due to the lack of economic resources in support of scientific research. For this, pathogens were characterized using some local small-scale epidemiological studies [1, 2], and when pathogens were dissected using national epidemiological surveillance, there is no sustained surveillance for the long term. Additionally, these advanced molecular techniques, compared to the traditional ones, are mostly needed in such breeding ground countries for multiple factors contributing to the infectious diseases spread and AMR complexity (lack of sanitation, war, refugees…) [3].

In this Ph.D. thesis work, we have reemphasized the importance of applying molecular techniques in clinical microbiological laboratories on three pathogens (*Shigella*, *Campylobacter*, and *Acinetobacter*). Even though these pathogens were previously tackled molecularly by different scattered Lebanese studies [4–6], our study was original owing to its novel perspective and generated data.

In our first study, we shed light on the identification problems of *Shigella* isolates in Lebanon, however, we were unable to draw the final identity of isolates. The study also adds more evidence on the phenotypic and genotypic closeness between *Shigella* and *E. coli* notably EIEC and the great need for a valid technique with a high resolution for such critical diarrheal etiologies endemic in developing countries. Additionally, WGS appears as a referee technique to evaluate the tested techniques and choose the most appropriate one with high specificity to be applied in laboratories with limited resources.

On the other hand, we revealed how rich data are from WGS of a *Campylobacter* isolate in Lebanon, a highly underestimated pathogen in the Lebanese clinical sector even with the increasing reports elucidating its epidemiology in the poultry sector [7, 8]. The WGS analysis facilitates the prediction of AMR determinants potentially explaining the high-level of phenotypic resistance, giving us the opportunity to further address many other microbiological aspects, such as the true species identity, virulence, and epidemiological backgrounds. Such

studies are quite interesting in the developing countries to prepare the progressive integration of WGS in their routine and to reach novel information, as well as new edges on underestimated pathogens with limited epidemiological data as *Campylobacter*. Despite that, the precipitously decreasing cost of WGS and the increasing number of user-friendly bioinformatics tools promise its wide integration as the future routine technique in the worldwide clinical microbiology labs, many other gaps related to deficiencies in the diagnostic capabilities should be plugged in countries with limited resources [9].

In the third study, we evidenced the potential of traditional molecular typing, namely MLST and PFGE, for CRAB isolates from the ICU of a tertiary care hospital. Although WGS provides the highest typing insights, such a traditional molecular approach maintained its importance and helped to clarify the clonal true nature of the outbreak and to delineate the clonal relationships between isolates. An efficient multifaceted control approach and good antimicrobial stewardship program allowed a full eradication of the outbreak and avoid the spread of new CRAB infections. For the routine perspective, typing should be done as possible in clinical microbiological laboratories to follow in real-time the epidemic trends of pathogens with outstanding outbreak ability. If the capacity of the clinical routine laboratory does not meet with high technical and financial demands of the molecular approach, isolates for notorious pathogens particularly MDR ones should be sent on a daily or weekly base to national reference laboratories to be typed at this moment by the traditional molecular typing methods pending for the wide integration of WGS in the routine typing. Accordingly, the requirement of national surveillance laboratories applying the molecular biology techniques rated to be an urgent necessity for both, clinical and foodborne pathogens. These groundbreaking national laboratories will perform the advanced molecular techniques, control outbreaks, and provide protective measurements in case of serious pathogen emergence. Therefore, the surveillance-based laboratories using molecular techniques could participate in elucidating the microbial outbreaks and in noticing the prevalence of infectious diseases.

Based on our aforementioned discussions, we recommend the needful application of molecular biology in routine laboratories in developing countries to identify pathogens, track antimicrobial resistance, and surveil the epidemic behavior. Meanwhile, we highly emphasize on the need for high-resolution techniques differentiating between closely related bacteria like *Shigella* and EIEC that are well-validated based on the large diverse genomic collection, to be applied for the sake of the international community. Finally, pending for a wide integration of

WGS in the routine laboratories, we must strengthen the capacity of the molecular biology facilities within the microbiological laboratories and this is done by standardizing laboratory procedures, training laboratory personal, installing equipment, and establishing a good waste management system.

REFERENCES

1. El Safadi D, Merhabi S, Rafei R, et al (2019) Cutaneous leishmaniasis in north Lebanon: re-emergence of an important neglected tropical disease. Trans R Soc Trop Med Hyg 113:471–476. https://doi.org/10.1093/trstmh/trz030
2. Haydar SM, Hallit SR, Hallit RR, et al (2019) Adherence to international guidelines for the treatment of meningitis infections in Lebanon. Saudi Med J 40:260–265. https://doi.org/10.15537/smj.2019.3.23965
3. Ismail MB, Rafei R, Dabboussi F, Hamze M (2018) Tuberculosis, war, and refugees: Spotlight on the Syrian humanitarian crisis. PLoS Pathog 14:e1007014. https://doi.org/10.1371/journal.ppat.1007014
4. Nawfal Dagher T, Al-Bayssari C, Chabou S, et al (2019) Investigation of multidrug-resistant ST2 *Acinetobacter baumannii* isolated from Saint George hospital in Lebanon. BMC Microbiol 19:29. https://doi.org/10.1186/s12866-019-1401-2
5. Rafei R, Al Kassaa I, Osman M, et al (2019) Molecular epidemiology of *Campylobacter* isolates from broiler slaughterhouses in Tripoli, North of Lebanon. Br Poult Sci. https://doi.org/10.1080/00071668.2019.1645945
6. Matar GM, Jaafar R, Sabra A, et al (2007) First detection and sequence analysis of the bla-CTX-M-15 gene in Lebanese isolates of extended-spectrum-beta-lactamase-producing *Shigella sonnei*. Ann Trop Med Parasitol 101:511–517. https://doi.org/10.1179/136485907X193860
7. Greige S, Rivoal K, Osman M, et al (2019) Prevalence and genetic diversity of *Campylobacter* spp. in the production chain of broiler chickens in Lebanon and its association with the intestinal protozoan Blastocystis sp. Poult Sci. https://doi.org/10.3382/ps/pez286
8. Talhouk RS, el-Dana RA, Araj GF, et al (1998) Prevalence, antimicrobial susceptibility and molecular characterization of *Campylobacter* isolates recovered from humans and poultry in Lebanon. J Med Liban 46:310–316
9. Ahmed SS, Alp E, Ulu-Kilic A, Doganay M (2015) Establishing molecular microbiology facilities in developing countries. J Infect Public Health 8:513–525.

https://doi.org/10.1016/j.jiph.2015.04.029