Article:
Krutova, M, Wilcox, MH orcid.org/0000-0002-4565-2868 and Kuijper, EJ (2019) A two-step approach for the investigation of a Clostridium difficile outbreak by molecular methods. Clinical Microbiology and Infection, 25 (11). pp. 1300-1301. ISSN 1198-743X

https://doi.org/10.1016/j.cmi.2019.07.022

Commentary

A two-step approach for the investigation of a Clostridium difficile outbreak by molecular methods.

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Clostridium difficile is a leading pathogen of healthcare-associated infections with an increasing incidence monitored between 2005-2013 in Europe [1]. In order to characterize C. difficile isolates, a wide range of molecular methods that target different regions in the C. difficile genome were developed [2]. The comparison of different typing approaches revealed differences in discriminatory power, turnaround time and cost [2]. According to the results from a survey on the typing capacity for C. difficile in Europe, the most widely applied method for characterisation of C. difficile isolates for surveillance purposes is PCR ribotyping (25 countries) followed by Multi-Locus Variable-number tandem-repeats Analysis (MLVA, 13 countries); Whole Genome Sequencing (WGS) was available only in 5 countries in 2017 [1].

The recent review "How to: molecular investigation of a hospital outbreak" written by Nutman and Marchaim proposed WGS as "the initial test and a single molecular test" for the characterisation of C. difficile isolates in a suspected outbreak situation [3]. Although WGS can be considered as an ultimate typing method [2], at present, it is usually used for fingerprinting, when comparing selected C. difficile isolates retrospectively, rather than prospective typing per se, probably because of its limited availability and high cost relative to more accessible methods. These obstacles could be the reason why healthcare institutions are reluctant to employ this method when dealing with a suspected C. difficile outbreak. Based on the experiences of three National reference laboratories for C. difficile, and recent publications of supporting data, we would like to propose a two-step approach for the investigation of a suspected CDI outbreak by molecular methods. This two-step approach would provide an epidemiological relevant timeframe for confirmation of a suspected CDI outbreak as well as being cost-effective by selecting highly related C. difficile isolates for further investigation.

Step one: The typing of C. difficile isolates by capillary electrophoresis polymerase chain reaction (PCR) ribotyping.

The capillary electrophoresis PCR ribotyping is recommended for characterisation of C. difficile isolates for CDI surveillance purposes, and indeed is the currently favoured approach by European centre for disease prevention and control (ECDC) [1]. This method is based on the variability in length and number of copies of intergenic spacer region (ISR) between genes encoding 16S and 23S ribosomal RNA. Using a validated protocol for capillary electrophoresis PCR ribotyping, a high inter-laboratory reproducibility of results is obtained [4]. PCR ribotyping enables the characterisation of a large number of C. difficile isolates in a short time to determine if a potential cluster of CDI cases, i.e. caused by C. difficile strain with the same ribotype, is present. When a common ribotype is identified among CDI cases with a strong epidemiological link (time and place), these C. difficile isolates should be investigated further using a more discriminatory, molecular fingerprinting method [5].

Step two: The subtyping of C. difficile isolates by Multi-Locus Variable-number tandem-repeats Analysis (MLVA) and/or Whole Genome Sequencing (WGS).

MLVA is targeted to variable regions in the C. difficile genome. The selected genome loci with short tandem repeats are amplified and separated by capillary electrophoresis. The difference between two isolates is defined as the sum of tandem repeats differences (STRD). A clonal complex (CC) was defined as STRD $\leq$ 2 and genetically related clusters are identified by an STRD $\leq$ 10 [6]. In C. difficile isolates of the same ribotype, MLVA enables the identification of CDI cases with indistinguishable, very highly related C. difficile strains, or unrelated CDI cases [5]. The technical requirements for MLVA are the similar to capillary-electrophoresis ribotyping, thus there is the capacity to introduce MLVA into laboratories where capillary-electrophoresis PCR ribotyping is available.

Using WGS, the comparison of genetic relatedness between C. difficile isolates is often based on single nucleotide variants (SNV) calling [7], when the genomic data of index C. difficile isolates are compared with a reference C. difficile genome. A more recent bioinformatics approach is core genome Multi-Locus Sequence Typing (cgMLST), where a defined set of C. difficile genes is analysed in order to determine the allelic profile of index C. difficile isolate [8]. The application of cgMLST for CDI surveillance seems to be an interesting alternative for laboratories that do not have the equipment for capillary electrophoresis PCR ribotyping and MLVA, but have access to the next generation sequencing platforms. Currently, the backward compatibility of cgMLST with PCR ribotyping is being investigated by a consortium of scientists. In addition to determining genetic relatedness between individual C. difficile isolates, WGS provides information on antimicrobial resistance determinants, mobile genetic elements and virulence factors (e.g. toxin genes).

The MLVA and WGS fingerprinting methods exhibit similarly high discriminatory power for C. difficile, although they target different parts of the genome [2, 7]; when compared in CDI outbreak/clusters settings, 95% concordance was observed [7]. For instance, through a combination of PCR ribotyping, MLVA and WGS, an outbreak and cross-national transmission of ribotype 018 was identified by in Germany [9].

Conclusion

For the purposes of retrospective epidemiologic investigations, C. difficile isolates or C. difficile positive stool samples should be stored [10] at microbiological departments of individual hospitals. When a C. difficile infection outbreak/cluster is suspected, the national or reference or central laboratory for C. difficile should be contacted [1]. For a molecular investigation of a suspected CDI outbreak/cluster, we recommend the use of capillary electrophoresis ribotyping as the initial short turnaround time typing method. When a

common C. difficile ribotype is identified, the second subtyping method, WGS or MLVA, should be applied.

Transparency declaration:

All authors report no conflict of interest relevant to this article.

No financial support was received for this study.

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