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Comparison of Molecular Methods of Microbial Serotyping

Archana Singh Sikarwar[1*] and Mariyam Nashtha Nashid[2]

[1]Faculty of Medicine and Health Sciences, Department of Human Biology, International Medical University, Kuala Lumpur, Malaysia.
[2]Department of Biomedical Sciences, International Medical University, Kuala Lumpur, Malaysia.

Author's contribution

This work was developed in collaboration by the both authors. Each author contributed equally to write the draft of the manuscript and managed literature searches and analyses of the study.

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Review Article

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ABSTRACT

Molecular serotyping methods have advantages and disadvantages in terms of different parameters. Since different methods depend on different parameters there is a possibility that one serotype detected by one method maybe missed by another method. So it is very important to use combination of molecular methods for serotyping because it would allow accurate serotyping. The best molecular serotyping method is pyrosequencing. However, advanced molecular methods, especially the sequence-based methods are currently evolving and relatively expensive; it would take some time for it to be widely used. To increase the serotyping capacity of Polymerase Chain Reaction (PCR), more serotype-specific primers should be designed. To increase the reliability of DNA microarray, an internal probe hybridization control (IHC) can be used which would indicate any variability in the hybridization process. Moreover to further decrease the cost of DNA microarray, all fluorescent labelling can be replaced with biotin labelling. Sequencing of more isolates of the same serotype would definitely improve the sequence-based serotyping assays.

Keywords: Serotyping; microbes; diagnosis; molecular methods.

**Corresponding author: E-mail: archana_sikarwar@imu.edu.my;*

1. INTRODUCTION

A serotype is a strain within a single species of microorganism such as bacteria or viruses which are differentiated based on the distinctive antigens on the microbe surfaces such as lipopolysaccharide, flagella and capsule. Serotyping is a definitive typing test that detects the distinct serotypes of a species. Serotyping is the main prediction for invasive disease potential and has a significant impact on the global health [1,2]. Serotyping is necessary for epidemiological surveillance since the distribution varies with age, time and geographical regions [3]. It determines the prevalence of serotypes, as some have the potential for lower antibiotic susceptibility [4]. Serotyping is used to determine the serotypes to be included in a vaccine such as in the case of *Streptococcus pneumoniae* [5]. It helps to monitor the serotype distribution after introduction of vaccine, to assess the long-term effectiveness of vaccines, monitor emergence of non-vaccine strains and ecologic impact of vaccines [3,6,7,8]. Continuous serotyping is crucial to monitor serotype replacement [9]. Serotyping allows early assessment of outbreaks and trace the origin [10]. Moreover it increases our knowledge in understanding the pathogens [1]. Traditionally serotyping was performed based on the agglutination reaction between the antigen of the microbe and specific antibody. However there is a vast number of disadvantages associated with conventional serotyping methods. Firstly it is time consuming, labour intensive, technical expertise requirement and expensive due to the use of large collection of antisera which is only generated by specialized laboratories with animal facilities [4,11,12]. The method is impractical for serotyping large number of samples [13]. It could generate equivocal results due to cross reactivity between different sero groups [11]. Some of the strains maybe left non-typeable due to masking of capsular antigens [14]. Furthermore it cannot type “rough” strains which lacks O-antigen due to a mutation [15,16]. In addition it also requires viable microbes and cannot be used to serotype culture of negative samples [17]. Antibiotic treatment at the beginning of the illness causes the serotyping more difficult [17]. Serotyping can be done by both phenotypic and genotypic methods [18]. However phenotypic methods such as slide agglutination or Quelleng reaction lack the discriminatory power [18]. To overcome all these difficulties of conventional serotyping as mentioned before, robust and discriminating molecular based or DNA based methods have been developed [14]. Molecular based serotyping methods are classified into three categories based on restriction analysis of DNA, PCR amplification of target gene and identification of DNA sequence polymorphisms [19]. Some of the molecular serotyping methods include multiplex real time PCR, DNA microarray, pulsed-field gel electrophoresis (PFGE), ribotyping, amplified fragment length polymorphism analysis, multi-locus sequence typing (MLST), pyrosequencing and ligation based microarrays [18,19]. An overview of some of these methods such as multiplex real time PCR, DNA microarray, pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and pyrosequencing with their respective performance, strengths and weakness are discussed below.

2. AMPLIFICATION / HYBRIDIZATION BASED METHODS

2.1 PCR-based Serotyping

The basic concept of PCR-based serotyping depends on the size difference between amplicons after amplification of the crude DNA template by serotype-specific primers [20,21,22]. PCR-based serotyping is carried out either by multiplex PCR where the primer sets are multiplexed in a single reaction to increase throughput or as real-time PCR for rapid analysis [19]. The first sequential multiplex PCR was developed by Pai and colleagues to detect the serotypes associated with invasive pneumococcal disease in America composing of 7 consecutive PCR reactions [21,22]. Researchers adapted and improved the original sequential multiplex PCR. The advantage of PCR is that, it can be adapted in such a way that group combinations of serotype-specific primer sets [21]. It allows the introduction of new serotypes in the scheme [21]. This is essential in cases where pneumococcal serotype distribution does not follow the normal pattern [21]. Conventional serotyping cannot be done on culture negative samples. However it has proven that PCR-based methods can be performed on the culture negative samples. A study was carried out to assess serotyping by multiplex sequential PCR (MS-PCR) and real-time PCR for invasive pneumococcal disease in a paediatric setting with 36 culture negative nasopharyngeal swab samples [16]. The potential to detect multiple pneumococcal serotypes were also evaluated [16]. Results are shown in Fig. 1.

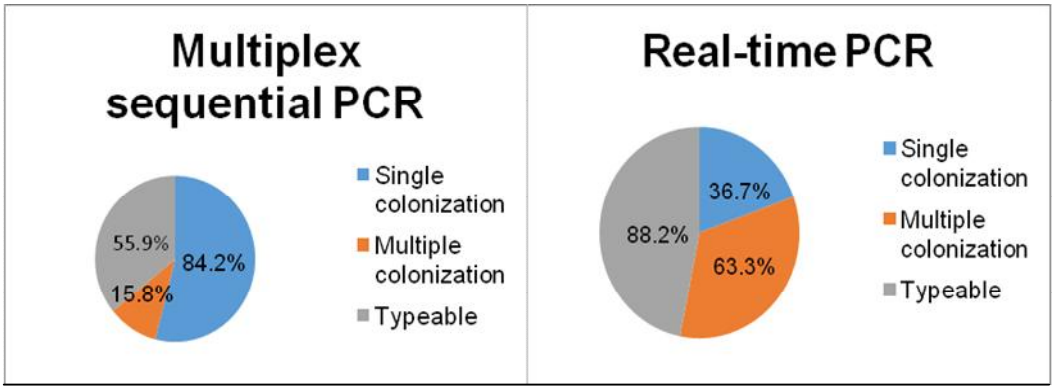

Fig. 1. Percentage of typeable samples showing single and multiple serotypes as proven by (A) multiplex sequential PCR and (B) real-time PCR [16]

Results showed that real-time PCR is more sensitive than multiplex sequential PCR because it can type more samples and detects more multiple serotypes compared to multiplex sequential PCR [16]. Multiple colonization studies give more information about the disease condition [16]. PCR-based serotyping has an advantage in resource-poor countries because it can performed on clinical samples directly [23]. This is much easier than bacterial isolates which needs rapid transportation to skilled laboratories [23]. Several studies have validated serotyping by PCR methods on blood samples, pleural fluids and cerebrospinal fluid [6,23,24,25]. One of the potential advantage of real-time PCR for serotyping compared to other PCR-methods is that, it is more rapid and there is a minimal chance of contamination of the amplicons because it is carried out in a closed system [26]. However in other PCR methods there is risk of amplicon contamination which would give inaccurate serotyping results [26]. Moreover the use of crude DNA template eliminates the use of specialized template preparation [27]. MS-PCR also allows the detection of most common serotypes using minimum number of PCR reactions [21]. The main drawback is inability to discriminate closely related serotypes such as 6A, 6B, 7A and 7F [21,25,28]. It identifies limited number of serotypes [27]. Moreover it could mislead the detection by giving positive results for rare strains that does not express capsule [28]. Secondary serotypes within a mixed culture cannot be readily detected by these methods [27]. Primer cross-reactions can occur and PCR based methods are not sensitive enough to serotype when the bacterial load is too low [21,29].

2.2 DNA Microarray

DNA microarray consists of a well plate of mini spots where probes are immobilized. Genomic DNA will be extracted from the microbe and the target gene will be amplified and labelled with a fluorescent dye. These targets will be hybridized with complementary probes and unbound fragments will be removed by washing. The microarray slide will be scanned using fluorescence microarray scanner and signals will be detected for each serotype [20,30,31,32]. Two types of probes can be used, which is either oligonucleotides or PCR products [30]. There is no significant difference in sensitivity between these two, however oligonucleotides are found more specific than PCR products [30,33]. The first model DNA microarray was developed to distinguish *Escherichia coli* serogroups *(*O7, O104, O111, and O157) provided successful results. [30] DNA microarray was compared with multiplex RT-PCR and real-time RT-PCR to serotype different foot-and-mouth-disease virus (FMDV) and swine vesicular virus (VSV) serotypes in 35 samples of calves inoculated with different serotypes of FMDV and VSV. [45] Microarray serotyping was done as indirect (post-PCR labelling) and direct (concurrent PCR labelling). Results are shown in Fig. 2 [34].

Results showed that microarray (indirect) is the most sensitive method out of all for serotyping these viruses because the number of samples correctly serotyped is highest for it. DNA microarray can also be used to detect dual infection with two dengue serotypes [20]. In another study to detect dengue serotypes, multiplex rRT-PCR has also shown to detect both serotypes in one sample [35].

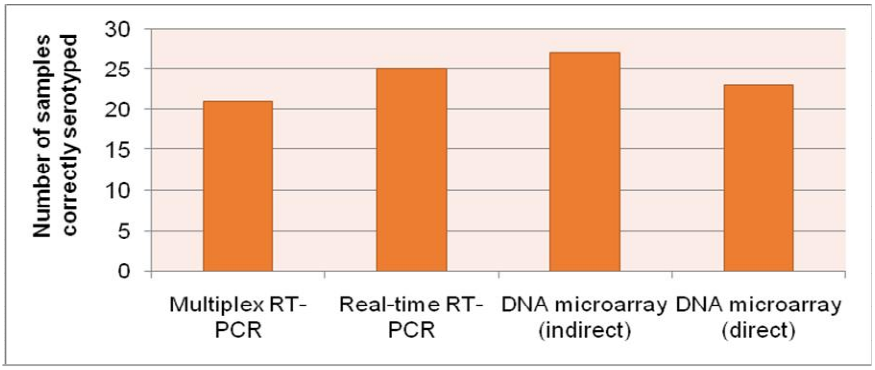

Fig. 2. Number of samples correctly serotyped by multiplex RT-PCR, real-time RT-PCR and DNA microarray as indirect and direct [34]

A DNA microarray was also used to develop a serogenotyping assay [36]. The benefit of genotype based serotyping is that it eliminates the phenotypic differences (R-forms which cannot be analysed by classical serotyping) [36]. DNA microarray was compared with RT-PCR and gel electrophoresis to type dengue serotypes in *Aedes* mosquito obtained from 35 cities in Brazil [20]. Result showed that DNA microarray could detect all serotypes in the infected mosquito pools. But RT-PCR and gel electrophoresis was unable to detect two serotypes in two mosquito pools, which showed DENV-1 by microarray [20]. However RT-PCR and gel electrophoresis showed specific amplicon for DENV-1 when the concentration of cDNA used was increased [20]. Microarray is highly specific, sensitive and reliable [37,38]. Microarray results are reproducible because the results are automatically read and recorded enabling further analysis [39]. Most importantly it has a high-throughput by testing a batch of isolates simultaneously [39]. Probes hybridize well with the targets with no detection of unspecific signals [20]. Moreover serotype-specific probe redundancy allows it to cover a diverse range of serotypes [34]. It is becoming a promising recent serotyping method for epidemiological surveillance and clinical diagnosis [40]. However with all these advantages it has certain drawbacks. The main drawback observed is false-positive signals among serotypes which are closely related having high sequence similarity [36,41]. Studies showed that microarray cannot differentiate *Salmonella* serogroup A ,D1, E1, E4, O67 and B.[36,37] Also *Legionella pneumophilia* serotype O8 from O14 and O12 from O15 [38]. This limitation can be overcome by adding more serotype-specific probes [41]. However serotype-specific probe designing is high-time work due to large number of genomic sequence available in public databases [42]. Furthermore somehow it could be technically demanding and costly due to expertise in construction of microarray chips [43,44].

2.3 Advantages of Microarray over PCR Methods

Microarray is a rapid method with high-throughput compared to PCR-based serotyping [32,38]. Multiple serotype detection in a single platform cannot be done by PCR serotyping, because it has to be performed for each serotype separately with primers specific for each target gene, Therefore PCR methods are slower than microarray [30]. Microarrays can be routinely used for multiplex serotype detection, however RT-PCR allows maximum from four to seven targets for multiplex detection [20]. In comparison with conventional PCR, microarray is more sensitive in detecting low levels of cDNA with a limit of 0.05-0.2 ng cDNA per spot, however on a standard gel it is approximately 20-30 ng [20]. Moreover for PCR methods, it is difficult to quantify PCR products and unable to differentiate amplicons of similar size from different serotypes [32,38]. Furthermore target-probe hybridization in microarray instead of gel electrophoresis used in PCR, can reduce the detection of non-specific sequences in amplified products. In addition, the concept of hybridization in microarray increases the use of multiple probes for detection of wide range of serotypes which would decrease false negative results occurs due to evolution of the microbe [34,43]. This is an advantage of microarray especially over real-time RT-PCR [34]. On the other hand, PCR methods can detect relatively small number of serotypes in a single assay and there is a difficulty in designing compatible primer sets [43].

3. RESTRICTION-BASED METHOD

3.1 Pulse Field Gel Electrophoresis (PFGE)

PFGE is a DNA fingerprinting method considered as the gold standard for subtyping various foodborne pathogens [19,45]. It is very useful during outbreaks and applicable for short term epidemiology [46]. For PFGE to perform, microbe are immobilized in agarose plugs and treated with enzymes and detergents to release the free DNA. After washing the agarose plugs to remove unwanted debris, purified DNA is digested with rare restriction enzymes specific for each microorganism. Agarose plug will be added on to the agarose gel to perform electrophoresis. The polarity of current will be changed at regular intervals allowing separation of DNA fragments. Gel will be visualized by a fluorescent dye and images will be captured for analysis. This is will which showed the fingerprint of that particular strain [19,46]. PFGE has a significant improvement compared to PCR-based serotyping. This is because immobilization of DNA will prevent from mechanical shearing and it generates genetic profile based on the whole genome but PCR selects a particular gene [19]. Studies show that PFGE has been used in successful typing of *Salmonella*, *Shigella*, *Leptospira*, *Yersinia* and *Escherichia* O157:H7 [19,45]. It has a high discriminatory power and reproducibility [19]. However it is expensive, labour intensive and time consuming taking 2-4 days [19]. Disadvantage of this method is that, if a mutation of the strain doesn't change the mobility of DNA on the gel, then it would not be identified as a separate serotype [19]. However using multiple restriction enzymes the discriminatory power can be increased [19].

4. SEQUENCE-BASED METHODS

4.1 Multi-locus Sequence Typing (MLST)

MLST method involves discriminating strains by comparing DNA sequences obtained from 7 housekeeping genes (genes for basic cellular function) which have low genetic variability [19,42,47]. This method is the gold standard for using in long-term (global) epidemiology [46]. It is highly reproducible and portable allowing electronic exchange of data between laboratories [46,48]. It has been successfully used to identify the serogroups of *Shigella* and is one of the best method for group B *Streptococcus* typing [47,49]. Other molecular methods such as PFGE and RFLP have disadvantages over MLST, because data cannot be compared across different laboratories and the results are too discriminatory for global epidemiology [50]. Housekeeping genes cannot distinguish strains which have undergone recent genetic change due to its low rate of genetic variability as mentioned before. So for a better choice virulence genes are used. Such MLST scheme is known as multi-virulence-loci sequence typing (MVLST) [19]. Many studies have shown the comparison of MLST with PGFE for discriminatory power. Some studies of *Vibrio cholera* and *E. coli* have shown that MLST has higher discriminatory power than PFGE. However some studies for *P. aeruginosa*, *S. aureus* and *E. coli* have showed that PFGE has higher discriminatory power [46,48]. These differences in results is due to the usage of housekeeping genes or virulence genes and the variation in number of loci sequenced [17,19]. The limitation of this method is that it is expensive, labour intensive and limited discrimination due to analysing genetic variation in a small part of genome (use of housekeeping genes) unlike PFGE [46,48]. Whole genome sequencing based molecular methods have developed to obtain a maximum discriminatory power among strains, replacing both MLST and PFGE.

4.2 Pyrosequencing

It is a non-electrophoretic real-time DNA sequencing method for short sequences (50-60 nucleotides) including SNPs [51,52]. It has the maximum discriminatory power required for accurate serotyping [17]. It is cost effective, rapid, high throughput, reproducible, user-friendly, minimum labour intensive, accurate, flexible and portable to share data between laboratories [52,53,54]. Another advantage of this method is that it can be reanalyse the raw data obtained from experiments when the serotyping database is modified [52]. It has been used to subtype HIV, hepatitis virus and influenza virus [55]. The major disadvantage of pyrosequencing is that it is limited to short DNA fragments and the analysis can be challenging and complex [53,56]. Studies have showed the potential of pyrosequencing assay to further discriminate serogroup 6 of *S. penumoniae* when PCR-based serotyping could not. It identified the SNP in the wciP gene and differentiated serogroup 6 into 6A and 6B [27,52]. Comparison of key features between conventional and molecular serotyping methods are shown in Table 1.

Table 1. Comparison of key features between conventional and molecular serotyping methods

Method	Parameters						
	Serotype identification capacity	Serotype discriminatory power	Reproducibility	Cost	Analysis time	Labour-intensive	References
Traditional	++	+	+	+++	+++	+++	1,10,11,12,13
PCR	++	+	+	+	++	++	21,25,26,28
DNA microarray	+++	+	+++	++	+	+	36,37,38,39
PFGE	+++	++	+++	+++	+++	+++	19
MLST	+++	++	+++	+++	+	+++	45,48
Pyrosequencing	+++	+++	+++	+	+	+	51,52,53,54

Key: Low: + ; Medium: ++ ; High: +++

5. CONCLUSION

Each of the molecular serotyping methods described have advantages and disadvantages in terms of different parameters. Since different methods depend on different parameters there is a possibility that one serotype detected by one method maybe missed by another method. So it is very important to use combination of molecular methods for serotyping because it would allow accurate serotyping [46]. In terms of all the parameters as stated before, the best molecular serotyping method described in this review is pyrosequencing. All these advanced molecular methods, especially the sequence-based methods are currently evolving and relatively expensive, it would take some time for it to be widely used. All these methods have proven highly valuable in serotyping. We hope in a near future we can obtain the maximum use of them after further developments been made to the methods.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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