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A REVIEW ON ADVANCED SEROTYPING METHODS FOR IDENTIFICATION OF *KLEBSIELLA PNEUMONIAE* CAPSULAR SEROTYPES

Archana Singh Sikarwar, Raevathi a/p omasanggar

* Human Biology Department, International Medical University, Kuala Lumpur, Malaysia
Email: archana_sikarwar@imu.edu.my

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Abstract

Identification of an organism plays an important role in diagnosis of diseases to understand the pathogenicity of that particular organism. Microorganisms differentiated by antigenic differences which are known as 'serotypes'. Liver abscess was recently recognized as a new invasive syndrome and serotyping serves as an important tool to distinguish strains of unusually virulent. During last ten years, highly reported incidents of liver abscess are of Klebsiella pneumonia. There are 78 capsular serotypes have been identified for Klebsiella pneumoniae which can be identified by using new advanced methods for serotyping of Klebsiella pneumoniae. Recent advanced capsular serotyping methods, more specifically molecular-based capsular serotyping methods. Even though, molecular serotyping has its limitation especially in sequencing, the limitations may be resolved with next generation sequencing (NGS). Advancement of serotyping is possible by development of convenient DNA microarray rapid kits to detect the highly virulent serotypes of Klebsiella. Pneumonia.

Introduction

Serotyping is one of the typing techniques used to identify microorganisms of same species that can vary in the antigenic determinants manifested on the cell surface. Microorganisms differentiated by antigenic differences are known as 'serotypes'. Liver abscess was recently recognized as a new invasive syndrome (1) and serotyping serves as an important tool to distinguish strains of unusually virulent. During the ten years (2004-2014), highly reported incidents of liver abscess are of *Klebsiella pneumoniae*.(1-13) Capsular serotype K1 (1-5,7-14) and K2 (6,7) are thought to be the major virulence determinants. Many incidents of liver abscess were reported with metastatic complications such as endophthalmitis, meningitis, necrotising fasciitis, endocarditis and other illnesses exclusively with serotypes K1 and K2.(2,3,6-9,11-14) Metastatic endophthalmitis can result in blindness if not treated within 24 hours with effective antibiotics(14). Therefore, early detection of microorganisms is necessary to provide early treatment and also further development of therapeutic agents. Capsular serotyping is the common method for detection of *K. pneumonia* serotypes. This is because capsule which is made up of complex acidic polysaccharides is considered the major virulence determinant of *Klebsiella*, and 78 capsular serotypes have been identified.(15) Conventional capsular serotyping method which uses antiserum broadly known to suffer from certain drawbacks. Two common conventional K-serotyping methods are capsular swelling technique (Quellung) and counter current immunoelectrophoresis (CIE). CIE is better than Quellung method in terms of the specificity, economical value, time consumption and reduced subjectivity.(16) Significant drawbacks in these two methods are cross reactions and untypability. Quality and specificity of antisera affect cross reactions (16) while noncapsulated strains are untypable by antisera (17). Moreover, the capacity of typability using antisera by CIE method is also inconsistent in different studies from as low as 63% to 90%. (18-22)CIE in particular is usually performed by few reference laboratories because of costly antisera and tedious procedures. (17) As a result of these limitations, capsular serotyping methods are slowly advancing to molecular level. This review herein provides an overview of the advances in capsular serotyping methods

Capsular serotyping by phenotypic methods

Double immunodiffusion and immunoblot analysis

Double immunodiffusion and immunoblot analysis are other serotyping methods besides Quellung and CIE that uses antiserum for serotyping. These methods are advanced in terms of technique of serotyping. Double immunodiffusion assay commonly used to type capsular K1 or for a detailed serotyping to reconfirm any initially tested serotype for antigenicity. (23-27)The principle of this method is testing antisera against extracted extracellular polysaccharide.(23) Result will be analyzed based on the pattern of precipitation line. (23) The disadvantage of this technique is time consuming for incubation, precipitation line analysis and staining procedure. This technique was compared in a study with modified immunoblot serotyping method to type only selected capsular serotype. (15) Both the techniques have been addressed with cross reactions (15) therefore low specificity similar to other methods using antiserum. These shows that even when different type of techniques is being employed to use antiserum for serotyping, complete specificity seem impossible. But the sensitivity of immunoblot analysis was high and reduced the consumption of antiserum than double immunodiffusion and CIE. (15) The principle of immunoblot method is blotted capsular extracts on nitrocellulose membrane will be tested against desired antisera and analysed using chemiluminescence property under X-ray film. (15) Limitation of immunoblot analysis is, it requires a lot of reagents which may be costly for a routine serotyping. Both double immunodiffusion and immunoblot analysis methods have not been evaluated for all the 78 strains of *K. pneumoniae*. This technique was used only for detailed serotyping to study certain important capsular types.

Molecular-based capsular serotyping methods

i) cps PCR- RFLP

Method was developed to identify the capsular serotypes of *Klebsiella* isolates without using antiserum.(17) In brief, the capsular loci responsible for capsular polysaccharide expression in all species of *Klebsiella* genus were amplified by PCR and digested with restriction enzyme HincII to generate profiles (C patterns) for all the strains. The generated C patterns were finally established into a database making it possible to detect serotypes without using antiserum.(17) This method can detect 75 of 77 known serotypes based on 75 distinct C patterns (97.4%).(17) This technique has a high discriminatory power than conventional K-serotyping because different strains of same serotype produced unique C patterns.(17) This could cause result interpretation difficult and require cps sequencing to distinguish.(15) But, it is known that severe liver abscess infection caused by *K. pneumoniae* are of serotypes K1/K2 but not all infections with K1/K2 serotypes result in liver abscess with metastatic infections. (1) This means only several strains of K1/K2 are virulent. Therefore, C patterns could probably used to identify the exact virulent strain. In addition to that, C pattern of K2 reference strain is significantly different than that of a K2 clinical strain obtained from abscess which was found during the development of this method.(17) This could be an insight to reference strains which are used to make antisera in the reference laboratories, may not, complement the strains which are causing diseases. Distinct C patterns were also produced for 3 of 4 noncapsulated strains.(17) When this method was applied to clinical isolates of *K. pneumoniae*, 82% of the isolates found to be discriminated from their C patterns. (17) The high stability of C patterns was proved by analysis of strains collected many years apart and different sources. (17) This is an added advantage for long term epidemiological studies. (17) A small percentage of strains (4.5%) were nontypable due to unsuccessful PCR amplifications but still outweigh the nontypeable capacity (8-23%) of traditional K serotyping. (17) In brief, this technique has improved typability rate and discriminatory power which are essential for a serotyping technique. There are limitations if it is to be applied for routine serotyping in diagnostic laboratories. Instruments used in this technique such as DNA purification kit and Expand Long Template PCR system can be costly depending on laboratories. Moreover, the overall procedure is complex and requires trained expertise. The amplification products expected to be very large (> 18kb) where diagnostic laboratories with normal PCR methods typically amplify DNA fragments of between 0.1 and 10 kb cannot perform the amplification unless equipped with costly equipments. Besides that, time taken for the overall procedure is long with at least 20 hours for RFLP analysis. Therefore, this technique may not be convenient for routine diagnostic procedures even though very useful.

ii) PCR of serotype- specific capsular ORF

Capsular polysaccharide synthesis loci of a bacterial genome has conserved and variable regions. Conserved region has ORFs responsible for capsular antigen translocation and capsule assembly while variable region involved in the capsular repeat unit synthesis and polymerization. (23) The mag A gene which has been recognized exclusively in K1 is located in the variable region (3,23,28-31) The second largest serotype to cause liver abscess and

complications is K2. (1) Capsular serotype K2 also has its serotype-specific region studied and ORF-9 or k2A is accepted as the capsular genetic determinant specific to K2.(32-34)

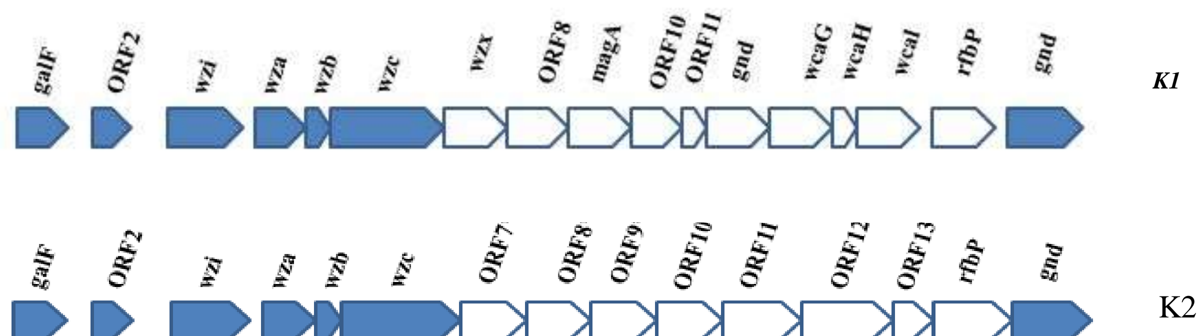

Figure 1.Capsular polysaccharide synthesis (cps) regions. This figure shows the comparison between capsular polysaccharide synthesis (cps) regions NTUH-K2044 (K1) and Chedid (K2). Open reading frames are shown in arrows. Blue arrows indicate the ORFs conserved in the serotypes while white arrows shows the ORFs of variable region. Some ORFs are labeled with gene names. ORFs without gene names are numbered as ORFs (ORF in K1 and ORF* in K2)

Other genotypes for K5, K20, K54 and K57 related to community-acquired invasive PLA syndrome were subsequently described.(23,35) As of December 2013, cps sequences of 12 capsular types have been published onto Genbank. (36) Basis of the PCR method is amplification of genetic regions using primers specific to genes such as magA and *k2A* for both K1 and K2. This method is more reliable, rapid and completely specific for the identification K1 and K2, exclusively useful in the incidents of liver abscesses given that K1 and K2 are the two major virulent strains. (28,33,35,37) The product size of PCR is also small(~ 2 kb) and therefore, manageable. This technique although found to be rapid and specific, PCR may not be needed to distinguish just few serotypes causing liver abscess unless otherwise happen in future. The same detection could be done by a simple antiserum Quellung method given that the quality of antiserum is high. Besides, for PCR, trained expertise will be needed to prepare primers to type each serotype. Lack of sequences for all other capsular serotypes limit the application of PCR to detect other serotypes.

Besides identifying serotype-specific genes, PCR is also employed to identify rmpA gene. The rmpA gene is known as a regulator for capsular polysaccharide synthesis which do not function independently but help in producing virulent capsules of K.pneumoniae. (1) It is not confined to particular serotype but when it is present, hypermucoviscosity phenotype can observed through cultures.(1) PCR approach to detect rmpA gene can be useful to directly identify virulent mucoviscous serotype from samples without the need to culture to confirm the hypermucoviscosity phenotype.

iii) Multiplex- PCR of serotype-specific capsular ORF

Availability of sequences of some capsular types of K.pneumoniae had led to a trial of multiplex PCR for a more rapid and accurate detection. The specificity of multiplex PCR is 100% as all the 6 reference strains were successfully detected by multiplex PCR. (35,38) Trial on clinical isolates also showed 100% specificity for K1, K2 and K5 and the same isolates when reacted with antiserum using CIE and Quellung showed few cross reactions.(38) The advantage of multiplex PCR is more specificity compared to CIE and Quellung. The method is rapid and simple. It is also cost effective since there is no requirement for antiserum preparation. This technique will be very useful if applied in routine serotyping since serotype K1 and K2 are most significant serotypes causing infections. Diagnosis can be made fast and metastatic infections from liver abscess can be prevented with rapid treatment. The main disadvantage of the multiplex is the kit for genomic DNA extraction can be costly. Limitation is clinical trial was done only for small numbers of serotypes that are frequent in causing bacteremia and liver abscess.

Identification of all the open reading frames of all 78 K serotypes is necessary but it is doubtful if all the serotypes can be detected through multiplex PCR with the limitations of PCR multiplexing.

iv) wzi sequencing

Wzi sequence based serotyping method was developed by Brisse et al with an aim to determine the capsular type for Klebsiella strains rapidly. (22) The wzi gene is one of the six genes in conserved region of cps locus of all capsular types of K.pneumoniae.(Fig. 1) The function of this gene is to produce an adhesive protein for attachment of capsule polysaccharide to the outer membrane.(22) Technically, the procedure for developing the sequences for all the serotypes is complex. However, the sequencing was worth done because all the K serotypes have unique wzi sequences except for nine serotypes. When the technique was evaluated with documented strains of clinical isolates, the typability rate was 98.1% while specificity was 98.3%. (22) This is very high compared to CIE where typability rate was 81% and specificity was 94.4%. (22)A reference wzi sequence database was created for all the K strains sequenced in the study.(22) Therefore, this technique is a simple and rapid method for any clinical strains to be identified given that it is virulent. There is no need for primers for each serotype because wzi region is a conserved ORF in cps region. In brief, sequencing the wzi gene product of PCR of a clinical strain will produce a sequence which can be referred to the database developed. Percentage of predicting the K serotype is 94%. (22) Limitation of the technique is there are still 9 serotypes could not be sequenced and should not be underestimated even though those serotypes known for not causing any serious illness.

v) wzc sequencing

wzc sequencing method was developed recently for wzc region. (36) The wzc gene (Fig. 1) functions to code for a protein responsible for capsule assembly. Seventy-six capsuler types have its wzc variable region sequenced and added into a database.(36) Reference strains of two capsular types which are K15 and K50 known to lack amplifiable wzc genes and were proven to be acapsular which is a new discovery.(36) It has high typability rate (96.55%) except for one single strain assumed as a new serotype.(36) Advantage of this technique is it has improved specificity and typability capacity compared to all other molecular serotyping methods discussed previously as well as serotyping using antisera. Again, primers need not to be developed for each serotype since wzc is a conserved region in cps region. Limitation is only regarding the sequencing of wzc region after PCR amplification. Therefore, this method is still technically complex similar to wzi sequencing for a routine serotyping.

Table 1: This table illustrates the comparison of the key attributes of some capsular serotyping methods of Klebsiella pneumoniae

Method	*Clinial Isolates typeability rate (%)*	*Serotype specificity (%)*	*Cost effectiveness*	*Analysis time*	*Total number of validated serotypes*	*References*
Capsular swelling	*81.1 - 90*	*63.9*	*Expensive*	*2 strains/hour*	*72*	*(16,39)*
Indirect immunofluorescence	*94.5*	*93.1*	*Expensive*	*1 strain/4 hours*	*72*	*(39)*
Coagglutination	*100*	*100*	*Moderately expensive*	*5 strains / <1.5 hours*	*5*	*(40)*
Latex agglutination	*100*	*100*	*Expensive*	*5 strains/ <1.5 hours*	*5*	*(40)*
CIE	*63 - 90*	*81 - 87.5*	*Moderately expensive*	*7 strains/hour*	*72*	*(16,19-21,41,42)*
Serotype-specific cps regions (PCR & Multiplex PCR)	*100*	*100*	*Moderately expensive*	*1 day*	*6*	*(35,38)*
cps PCR- RFLP	*95.5*	*97.4*	*Moderately expensive*	*2 days*	*77*	*(17)*
Wzi sequencing	*98.1*	*98.3*	*Moderately expensive*	*1 day*	*77*	*(22)*
Wzc sequencing	*100*	*96.55*	*Moderately expensive*	*1 day*	*78*	*(36)*

Conclusion

There are so many advanced capsular serotyping methods are available, more specifically molecular-based capsular serotyping methods. Even though, molecular serotyping has limitation especially in sequencing, the limitations may be resolved with next generation sequencing (NGS). For further advancement of serotyping method, researches may be conducted in development of convenient DNA microarray rapid kits to detect the highly virulent serotypes of *K. pneumoniae*. Further studies, are necessary supporting the molecular-based serotyping to find a gold standard serotyping method for *K.pneumoniae*.

Conflict of interest

None

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