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Molecular Diagnosis of Infectious Diseases: Past, Present and Future

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INTRODUCTION

Infectious diseases are common diseases all over the world. A recent World Health Organization report indicates that infectious diseases are now the world's biggest killer of children and young adults. The death of 45% in all and 63% in early childhood are caused by infectious diseases in nonindustrialized countries (WHO, 1999). In developed countries, the emergence of new, rare or already-forgotten infectious diseases, such as HIV/AIDS, Lyme disease and tuberculosis, has stimulated public interest and inspired commitments to surveillance and control (van Dissel, 1999). Recently, it is reported that infectious diseases are responsible for more than 17 million deaths worldwide each year, most of which are associated with bacterial infections (Picard and Bergeron, 2002). Hence, the control of infectious diseases control is still an important task in the world.

Infectious diseases are diseases caused by microorganisms including viruses, bacteria, fungi and parasites. The causal organisms and their relevant infectious diseases differ from decade to decade and from century to century. When some old diseases are controlled or even eliminated, for example, poliomyelitis, smallpox, some other new diseases appear, such as AIDS, Lyme disease and SARS. Some of the more controlled diseases, like tuberculosis, come back again and again for several reasons. According to WHO's report in 1999, six diseases cause 90% of infectious disease deaths, including pneumonia, tuberculosis, diarrhoeal diseases, malaria, measles and more recently HIV/AIDS.

To control infectious diseases, the first important thing is to make a correct and rapid diagnosis, especially for some acute infectious diseases, such as meningitis and pneumonia. Over the past century, clinical microbiologists have developed several different detection methods for microbial identification based on morphology and other variables. Some of them are still been using as standard diagnostic methods today, for example, Gram stain as well as microorganism culture. Molecular biological techniques start a new century for clinical microbial detection. During the last two decades, molecular based detection methods have been becoming more and more powerful tools in clinical diagnosis (Weisburg, *et al*., 1991; Widjojoatmodjo, *et al*., 1994; Fredricks and Relman, 1999).

This review is a brief presentation of the development and the current knowledge, literature and recommendation about the laboratory diagnosis of infectious diseases. The focus of this review is concentrate on bacterial infection and viral, fungal and parasitic infections are beyond the scope of this review.

INFECTIOUS DISEASES

In 1676, Anton van Leeuwenhoek, a Dutch cloth merchant and amateur lens grinder, first observed living microorganisms using his simple microscope, which he called animalcules. He examined animalcules in the environment, including pond water, sick people and even his own mouth and found that the animalcules existed everywhere. He described and recorded all the major kinds of microorganisms: protozoa, algae, yeast, fungi and bacteria in spherical, rod and spiral forms (Bobell, 1932; Porter, 1976). His discoveries opened up a new world namely the microbial world and this was the first milestone in the history of microbiology.

Although the suggestion that disease was caused by invisible living creatures was made by the Roman physician Girolamo Fracastoro in 1546, people did not clearly recognise the role of microorganisms in diseases, until 1876, after 200 years that Leeuwenhoek found his little animalcules, the Germanphysician Robert Koch established his famous Koch's postulates according to the relationship between *Bacillus anthracis* and anthrax. Koch's postulates include:

- In order to prove that a certain microbe is the cause of a certain disease, that same microbe must be found present in all cases of the disease.

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- This microbe must then be completely separated from the diseased body and grown outside that body in a pure culture.
- This pure culture must be capable of gibing the disease to healthy animals by inoculating them with it.
- The same microbe should then be obtained from the animals so inoculated and then grown again in a pure culture outside the body.

Koch was a great pioneer in medical microbiology and his postulates are still considered fundamental to bacteriology even today.

As Leeuwenhhoek described, microorganisms live in almost everywhere. In fact, only a minority of them cause disease. The microorganism which can cause disease is called a pathogen. A disease caused by a microbial pathogen is called an infectious disease. Causative pathogens include bacteria, fungi, viruses and protozoa. Infectious diseases can be classified into two different types: acute and chronic infectious diseases. Acute infectious diseases develop very fast, in just a few days or few weeks. In some of acute infectious diseases, the progress is so fast that patients will die in 24 h if they cannot get accurate diagnosis and effective treatment in time, for example, acute bacterial meningitis. Chronic infectious diseases develop more slowly, generally for a few months, a few years or even for a few decades, for example, tuberculosis and hepatitis B. Some infectious diseases can spread from person-to-person by different routes, such as direct person-to-person transfer, respiratory transmission, parenteral inoculation, sexual or mucosal contact and insect vectors, which are called communicable diseases. Communicable diseases can occur in populations and cause epidemics or even pandemic problems. Some outbreak diseases are so serious that they can cause hundreds, thousands, even millions of deaths in epidemic populations. For example, bubonic plague, caused by *Yersinia pestis*, spreading from Asia and carried by rat-fleas via the ports of the Black Sea to Europe, caused 42 million deaths, 25 million of them in Europe, in just less than five years between 1347 and 1352 (Karlen, 1995); Viral influenza, caused by *Influenza virus*, started in America in 1918 and then spread to the whole globe, causing about 675,000 (in Americans) deaths and 30 to 40 million death in the worldwide (Collier, 1996).

In 1796, Edward Jenner, a British doctor, developed a vaccine with milder cowpox virus to prevent the life threaten disease-smallpox. This was the beginning of the fight to infectious disease by using vaccines. Since then, during the last 200 years, several different vaccines have been used to prevent different infectious diseases and some of these diseases are now dramatically under control. Good examples include smallpox and poliomyelitis. Smallpox is caused by the smallpox virus. Over the centuries, naturally occurring smallpox, with its case-fatality rate of 30% or more and its ability to spread in any climate and season, has been universally feared as the most devastating of all the infectious diseases. Since the cowpox vaccine was used, the disease was reduced year by year. Finally, smallpox officially was declared eradicated by the World Health Organization (WHO) in 1980. Poliomyelitis is a central nerve system infection caused by poliovirus and is spread by contaminated faecal material occurring in young children under five and can cause total paralysis in just a few hours. Because the vaccines were used broadly in the last decades, the number of cases has fallen by over 99%, from an estimated 350 000 cases in 1988 to 1919 reported cases in 2002 (as of 16 April 2003). In the same time period, the number of polio-infected countries was reduced from 125 to 7. Some other infectious diseases, such as measles, rubella through the use of effective vaccines are under control.

However, we are now facing some new challengers: some diseases have re-emerged; and some new diseases which we never knew before have appeared recently to make new threats to humanity. For instance, tuberculosis (TB), a very old disease discovered by Robert Koch in 1882, was well controlled by the middle of last century. However since the 1980s, the disease has re-emerged because of antibiotic resistance species emerging and AIDS patients increasing. According to a recent WHO infectious disease report, two billion people worldwide are carriers of the tuberculosis bacillus. Every year, about 8.8 million people develop active TB and 1.7 million die of the disease; 99% of all TB sufferers live in developing countries (WHO, 2002).

Human immunodeficiency virus (HIV) was obtained from peripheral blood mononuclear cell samples collected in 1981 from seven HIV-infected U.S. individuals (Hymes, *et al*., 1981). The disease caused by HIV called Acquired Immure Deficiency Syndrome (AIDS). Although the disease was first reported in 1981, it developed so fast that in a short time, it occurred in a number of different countries (Masur, *et al*., 1981; Vilaseca, *et al*., 1982; Rozenbaum, *et al*., 1982). It is said that, there are 42 million people living with HIV/AIDS worldwide. It is estimated that a total of 3.1 million people died of HIV/AIDS related causes in 2002 (WHO, 2002). Another new infectious disease is the Severe Acute Respiratory Syndrome

(SARS) which has just occurred in South of China in November 2002. From the Guangdong province in China, the SARS virus travelled in humans to 30 countries in several areas of the world. The pattern of transmission in SARS is believed to be due to an imported hospitalized SARS case infected health care workers and other patients who in turn infected their close contacts and then the disease moved into the community. The disease was so new that before people realised it was a communicable disease, some of health care workers who looked after the sick patients had been infected. In affected areas, approximately 20% of all cases were in health care workers. Just in a few months, about 8500 people had been affected and more than 800 died from SARS (Nie, *et al.,* 2003). Such a new disease gives us a strong warning namely that humanity is in danger of attack by new infectious diseases all the time. At the beginning of the new century, Pollard and Dobson described fifteen infectious diseases including influenza, HIV, malaria and so on, which are now beginning to emerge in the 21st century (Pollard and Dobson, 2000). All these are diseases we have already known about. It is very possible that some diseases we do not know will emerge suddenly, like SARS.

In 1928, Sir Alexander Fleming discovered the antibacterial effect of penicillin. This discovery started a new era for the treatment of infectious diseases. Since then, the mortality of infectious diseases has been greatly reduced. For example, before the discovery and use of antibiotics, the case fatality rate of meningococcal diseases was about 70% of cases; currently, this rate is now just about 5% to 10%. To give patients effective treatment, the first thing that should happen is to get an accurate and rapid diagnosis. Generally speaking, to make a diagnosis for a disease should depend on four factors: patient's history, symptoms, signs and adjunctive data including laboratory detection. For infectious diseases, laboratory detection is aimed to identify causative pathogens, which plays a key role in diagnosis. As the laboratory detection is so important in the diagnosis of infectious diseases, over the last century, people developed many different detection methods and some of them, such as the Gram stain, microbial culture and immunologic detection, are still used today. Following the development in science, some new detection methods are coming out time to time, especially molecular-based detection methods which are used more and more widely in laboratory diagnosis. Here we just have a brief overlook the development and advantages and disadvantages about the conventional and molecular detection methods.

COVENTIONAL DETECTION METHODS

Conventional detection mainly depends on the phenotypic characteristics of detected microorganisms such as the bacterial isolate's morphology and metabolic capabilities. The targets of detection are the products of microorganisms, such as enzymes and antibodies. Such detection methods include: Gram stain, cultivation biochemistry identification and immunologic detections.

Gram stain: Bacterial staining techniques commenced in 1869. H. Hoffman stained bacteria in suspension with vegetable stains and observed the wet bacterial preparations under the microscope. In 1875, Karl Weigert, stained bacteria using various simple stains including the synthetic dye methylene blue. The simple stains clearly demosteated the shape and size of the organisms, but they could not identify the organisms. To solve this problem, Hans Christian Gram, a Danish bacteriologist, developed a new stain method in 1884, which was named with his name, namely the Gram stain.

The primary stain of the Gram's method is crystal violet. Those microorganisms that retain the crystal violet-iodine complex appear purple brown under microscopic examination. These microorganisms that are stained by the Gram's method are commonly classified as Gram-positive. Others that are not stained by crystal violet are referred to as Gram negative and appear pink red. Gram staining is based on the ability of the bacteria cell wall to retain the crystal violet dye during solvent extraction. The cell walls of Gram-positive microorganisms have a higher peptidoglycan and lower lipid content than Gram-negative bacteria. Bacterial cell walls are stained by the crystal violet. Iodine is subsequently added as a mordant to form the crystal violet-iodine complex so that the dye cannot be removed easily. This step is commonly referred to as fixing the dye. However, subsequent treatment with a decolorizer, which is a mixed solvent of ethanol and acetone, dissolves the lipid layer from the Gram-negative cells. The removal of the lipid layer enhances the leaching of the primary stain from the cells into the surrounding solvent. In contrast, the solvent dehydrates the thicker Gram-positive cell walls, closing the pores as the cell wall shrinks during dehydration. As a result, the diffusion of the violet-iodine complex is blocked and the bacteria remain stained.

Although the method has been established over one hundred years, Gram staining is still one of the most important staining techniques in microbiology today. It is almost always the first test performed for the identification

of bacteria because the method is simple, rapid, accurate and inexpensive for detecting bacteria and inflammatory cells in clinical specimens. In addition to Gram stain, some other special staining methods are used in diagnostic microbiology, including:

- Acid-fast staining for detecting *Mycobacterium* sp.
- Negative staining for detecting bacterial capsules such as *Neisseria meningitides* and *Streptococcus pneumoneae*
- Spore staining for detecting bacterial spores such as *Bacillus anthracis* and *Clostridium tetani*
- Flagella staining for detecting bacterial flagella such as *Salmonella typhi* and *Vibrio cholera*

Microbial culture: In 1860, Louis Pasteur, a famous chemist and probably the greatest biologist of the nineteenth century and father of microbiology, published the first semi-synthetic medium designed for the growth of bacteria. The medium consisted of ammonium salts, yeast ash and candy sugar. This was the beginning of bacterial culture. In 1872, Ferdinand Cohn, a famous German botanist and bacteriologist, published the recipe for a similar medium that he had devised. All these early media were liquid and it was then very difficult to obtain pure cultures from the liquid media (Collard, 1976). In 1880, Robert Koch found a new way to separate the mixed microbes and get a pure culture by chance-using solid media. He developed this method by using sterile surfaces of cut, boiled potatoes first and then using gelatine medium and successfully obtained different pure bacterial colonies. The weak points for the gelatine medium were that it was digested by many bacteria and melted when the temperature rose above 28°C. To overcome such problems, Dr Walther Hesse, one of Koch's assistants and his wife Fannie Eilshemius Hesse replaced gelatine with agar-agar and got a new culture medium-agar medium (Hitchens and Leikind, 1938). By 1882, Koch isolated the bacillus the caused tuberculosis and in 1887, Richard Julius Petri, a German bacteriologist, developed a new culture medium container,-namely the Petri dish, named after him. The Petri dish is one of the most universally used pieces of equipment in bacteriological laboratories today.

Presently, bacterial culture is still a single important detection method in clinical diagnostic laboratories. The diagnosis of infectious diseases is mainly based on the isolation and identification such etiological agents from clinical specimens. Growth media can be used in either liquid (broth) or solid (agar) phase or both depending on the kind of clinical specimen and the expected bacteria. Liquid media provides greater sensitivity for the isolation of small numbers of microorganisms, however, identification of mixed cultures growing in liquid media requires subculture onto solid media so that isolated colonies can be processed separately for identification. There are four different culture media used in diagnostic laboratory:

Supportive media: Containing nutrients that support growth of most non-fastidious organisms without giving any particular organism a growth advantage, for example, Blood agar and Columbia agar.

Enrichment media: Containing specific nutrients required for the growth of particular bacterial pathogens that may be present alone or with other bacterial species in a patient's specimen. This media type is used to enhance the growth of a particular bacterial pathogen from a mixture of organisms by using nutrient specificity. For example, the employment of chocolate agar for g *Neisseria* sp. and *Haemophilus* sp.

Selective media: Containing one or more selective agents, such as dyes and antibiotics, which are inhibitory to all organisms except those being sought. For example, Mannitol salt agar for the selective isolation of staphylococci and Camy-blood agar for selecting for *Campylobacter* spp.

Differential media: Employing some factor or factors that allow colonies of one bacterial species or type to exhibit certain metabolic or culture characteristics that can be used to distinguish them from other bacteria growing on the same agar plate. For example, MacConkey agar, which is used for the isolation and differentiation of lactose-fermenting and non-lactose-fermenting enteric bacilli.

Apart from culture media, culture conditions such as temperature, oxygen, carbon dioxide and culture time, also can be used to detect bacteria. For example, the best culture temperature for most pathogens is 37°C, but for most environmental bacteria it is under 30°C and for *Campylobacter* spp it is 42°C; Most bacteria need oxygen when they are growing, but some anaerobic bacteria like *Clostridium tetani* only can grow without oxygen; *Neisseria meningitides* only grows well in 5-10% CO_2 (micro aerophilic conditions) and most bacteria can grow well in 24 h, however some bacteria need a few days or even a few weeks to grow, such as *Mycobacterium tuberculosis*.

Microbial culture media are improving and developing all the time. In recent decades, as antibiotic-resistant bacteria are increasing quickly, (Arbuckle, 1968; Robinson, *et al.*, 1979; Butaye, *et al.*, 1999; Uyttendaele, *et al.*, 2001; Blanc, *et al.*, 2003), there are many reports relating to new media for their selector, as well as the detection of some new bacteria (Baylis, *et al.*, 2000; Moore, *et al.*, 2003; Siddique, *et al.*, 2003).

Biochemical identification: Biochemical identification is based on the characteristics of metabolism and physiology in different bacteria. The study of bacterial metabolism and physiology commenced from Louis Pasteur in 1857 and developed fast from the beginning of the twentieth century and has continued to the present. Over the last one hundred years, several scientists, such as Ferdinand Cohn, Martinus Beijerinck, the Dutch bacteriologist, Sergie Winogradsky, the Russian microbiologist, performed several studies and revealed several important aspects of bacterial metabolism and physiology and developed many new bacterial biochemical identification methods. The most famous names in this field perhaps are those of Marjorie Stephenson who elucidated many of the energy-yielding pathways in her laboratory in Cambridge and Albert Kluyver of Delft who had the genius to see the underlying unity in the diversity of microbial energy-yielding mechanisms.

Currently, biochemical detection methods used in clinical diagnostic laboratory include single enzyme tests and tests for the presence of metabolic pathways.

Single enzyme tests

Catalase test: The enzyme catalase catalyses the release of water and oxygen from hydrogen peroxide (H_2O_2+catalase=H_2O+O). Some of Gram-positive bacteria, for example, staphylococci, contain this enzyme. This test is for identifying Gram-positive bacteria.

Oxidase test: Cytochrome oxdase participates in electron transport and in the nitrate metabolic pathways of certain bacteria. The test is initially used for differentiating between groups of Gram-negative bacteria. Among the commonly encountered Gram-negative bacilli, *Stenotropomonas maltopilia* and *Acinetobacter* spp. are oxidase-negative, whereas many other bacilli, such as *Pseudomonas* spp. and *Aeromonas* spp., are positive. This test is also a key reaction for the identification of *Neisseria* spp. (oxidase-positive).

Indole test: Bacteria that produce the enzyme tryptophanase are able to degrade the amino acid trypophan into pyruvic acid, ammonia and indole. Indole is detected by combining with an indicator, aldehyde, that results in a blue colour formation. This test is used in numerous identification schemes, especially to presumptively identify *Escherichia coli*, the Gram-negative bacillus are commonly encountered in diagnostic bacteriology.

Urease test: Urease hydrolyzes the substrate urea into ammonia, water and carbon dioxide. The presence of the enzyme is determined by the inoculation of an organism to broth or agar that contains urea as the primary carbon source and followed by the detection and production of ammonia. The urease test helps to identify certain species of *Enterobateriaceae*, such as *Proteus* spp. and other important bacteria such as *Corynebacterium urealyticum* and *Helicobacter pylori*.

PYR test: The enzyme L-pyrroglutamyl-aminopeptidase hydrolyzes the substrate L-pyrrolidonyl-β-naphthylamide (PYR) to produce a β-naphthylamine. When the β-naphthylamine combines with a cinnamaldehyde reagent, a bright red colour is produced. The PYR test is particularly helpful in identifying Gram-positive cocci such as *Sreptococcus pyogenes* and *Enterococcus* spp., which are positive, while other streptococci are negative.

Hippurate hydrolysis: Hippuricase is a constitutive enzyme that hydrolyzes the substrate hippurate to produce the amino acid, glycine. Glycine is detected by production of a deep purple colour with ninhydrin. The hippurate test is most frequently used in the identification of *Streptococcus agalactiae*, *Campylobacter jejuni* and *Listeria monocytogenes*.

Tests for presence of metabolic pathways

Oxidation and fermentation tests: Bacteria use various metabolic pathways to produce biochemical building materials and energy. Most of the relevant bacteria mainly utilize carbohydrates (e.g., sugar or sugar derivatives) and protein substrates as sources of carbon and nitrogen. Determining whether substrate utilization is an oxidative or fermentative process is important for the identification of several different bacteria. The commonly employed tests in clinical laboratory include sugar fermentation tests, Voges-Proskauer test (VP Test) and the methyl red test.

Amino acid degradation: Determining the ability of bacteria to produce enzymes that either deaminate, dihydrolyze, or decarboxylate certain amino acids is often used in identification schemes. The amino acid substrates

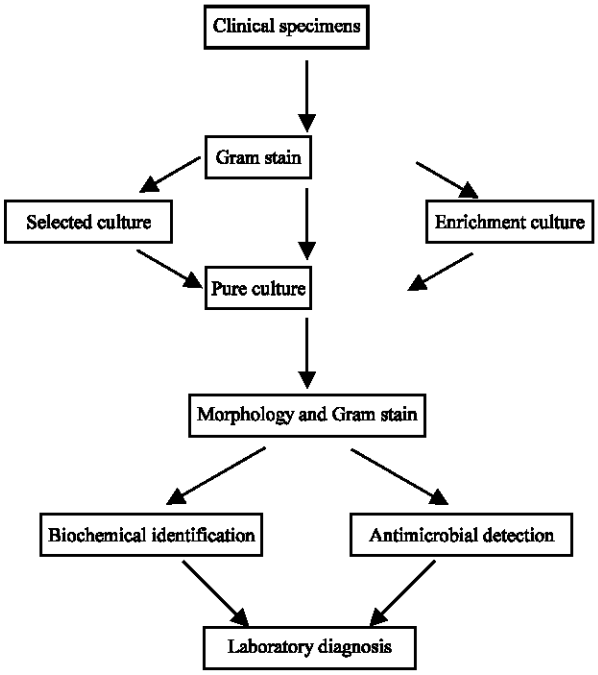

Fig. 1: A general algorithm of bacterial identification

most often tested include lysine, ornithine, arginine, phenylalanine and tryptophan cleavage (indole test).

Single substrate utilization: Some organisms can grow in the presence of a single nutrient or carbon source and this provides useful identification information. Such tests entail inoculation of organisms to a medium that contains a single source of nutrition (e.g., citrate, malonate, or acetate) and, after incubation, observing the medium for growth. For example, the citrate utilization test can identify *Citrobacter* which only can use citrate as carbon source.

Since 1970s, some conventional detection methods have been developed commercially (Stager and Davis, 1992). The commercial systems replaced compilations of conventional test media and substrates prepared in-house for bacterial identification. Presently, the API Systems are the commonly used systems in clinical diagnostic laboratories and are considered to be the gold standard systems (O'Hara, Rhoden and Miller, 1992).

Overall, a general algorithm of bacterial identification is showed in Fig. 1.

Immunological detection methods: Immunologic detection methods are based on the characteristic that antigens and antibodies can combine with each other in highly specific ways to detect microorganisms directly or indirectly. Antigens are foreign substances, usually high-molecular-weight proteins or carbohydrates, such as the components of the invading microorganism's structure, including cell walls and flagella, or their chemical products, like enzymes and toxins. Antibodies, also referred to as immunoglobulins, are specific proteins produced by certain cells in response to the presence of antigens in a human or animal host. Antibodies specifically attach to the relevant antigens at the antigen binding site and aid the host in removing the infectious agents.

In 1896, Max von Gruber, the Austrian bacteriologist and hygienist and Herbert Edward Durham, an English bacteriologist discovered bacterial agglutination. Durham first briefly reported the phenomenon of specific agglutination of antibodies against paratyphoid bacteria in the patient's serum and then it was described by both of them. In the same year, Georges Fernand Isidore Widal, the French physician and bacteriologist, published an account of the use of the agglutination reaction as a diagnostic aid in cases of suspected typhoid fever. This is the famous Widal's Test. It was mainly used for indirect confirmation of salmonellosis (as against typhus abdominalis, paratyphus and brucellosis), but also for diagnosing infectious diseases like shigellosis, tularaemia and spotted fever. Widal's Test was the beginning of detecting microorganisms using immunological methods.

Over the last one hundred years, immunological detection methods have developed quickly. For detection of microbial antigens, particle agglutination and enzyme immunoassays are now the most frequently used techniques in the clinical laboratory.

Particle agglutination: Particle agglutination is often used in clinical laboratory and includes latex particle agglutination (LA) and coagglutination. The principle of LA is that an antibody to a specific antigen is initially bound to the surface of latex (polystyrene) beads, then the antigen present in a specimen binds to the combining sites of the antibody exposed on the surfaces of the latex beads, forming visible cross-linked aggregates of latex beads and antigen. Coagglutination is similar to LA but the antibody is bound to a heat-killed and treated protein A-rich strain of *Staphylococcus aureus* to produce agglutination. Particle agglutination can be used to detect different bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis* and *Campylobacter jejuni* (Hill, *et al.*, 1994; Greenberg, *et al.*, 1995; Singhal, *et al.*, 1996; March, *et al.*, 2000; Chattopadhyay, 2002).

Enzyme-linked immunosorbent assay (ELISA): There are several approaches to ELISA, the one most frequently used for the detection of microbial antigens uses an antigen-specific antibody that is fixed to a solid phase,

which may be a latex or metal bead or the inside surface of a well in a plastic tray. Antigen present in the specimen binds to the antibody. The test is then completed by adding a second antigen-specific antibody bound to an enzyme that can react with a substrate to produce a colored product. When the enzyme-conjugated antibody is added, it binds to previously unbound antigenic sites and the antigen is, in effect, sandwiched between the solid phase and the enzyme-conjugated antibody. The reaction is completed by adding the enzyme substrate. ELISA systems were first developed during the 1960's and are still broadly used in clinical laboratories today. The methods can be used to detect bacteria, fungi, viruses in different clinical specimens (Holmberg, *et al*, 1985; Galgiani, *et al*, 1996; Kato, *et al*, 2001; Hunfeld, *et al*, 2002). Some commercial systems are available for the detection or different antigens, which make the methods simple and easy to control (Whittier, Holmberg, *et al*, 1993; Malan, *et al*, 2003). In recent years, ELISA is linked to Polymerase Chain Reaction (PCR-ELISA), have increased the detection sensitivity by 100-1000 fold (Muramatsu, *et al*, 1997; Gilligan, *et al*, 2000; Guiver, *et al*, 2000; Hong, *et al*, 2003).

Advantages and disadvantages of conventional methods: After over one hundred years development, conventional detection methods have been stable and the standard methods for laboratory diagnosis. However when combined with modern techniques, e.g., computer techniques and commercial systems, such methods become more useful, although they still have some scientific weaknesses.

Advantages

Simple and easy: Conventional detection methods do not need complicated and special techniques, although some methods do need experience, such as the Gram stain and they are still easy to learn and perform. After commercial systems are available, the performance, result analysis and quality control of the detection methods have now become simpler and easier for laboratory workers.

Low cost: As there is no need of expensive equipment and reagents, the costs of conventional detection methods are much lower than the costs of other detection methods. For example, for one sample, conventional detection methods only need about two pounds, but molecular detection methods will need about ten pounds.

Suitable for large throughput: As such the methods are easy to handle and available for large number of specimens at same time, conventional detection methods are suitable for use in hospital diagnostic laboratories. For example, a blood culture machine can contain 240 slots of BacTAlert module at meantime, but the largest PCR machine can only analyse 96 samples at any one time.

Provides an organism for antibiotic susceptibility testing: Conventional detection methods can provide an organism for antibiotic susceptibility testing for clinical doctors directly.

Disadvantages

Sensitivity and specificity limited: Generally, the Gram stain is most reliable at detecting $\geq 10^5$ bacteria per mL of body fluid (Coovadia. and Naidu, 1985; Balows, *et al*, 1991; Mahon, C. R. and Manuselis, 2000). Immunologic tests such as latex agglutination also are reliable only at detecting $\geq 10^5$ cfu per ml (Bingen, *et al,* 1990; La Scolea, L., Jr. and Dryja, D., 1984). If bacterial concentration in a clinical specimen is too low, Gram stain and latex agglutination will give a false negative result. The Gram stain also is related with some factors, such as pH, bacterial culture time, stain time. If the stain conditions are incorrect, it will give a incorrect result. Immunological detection is based on specific combination between antibody and relative antigen, i.e., the detection needs prepared specific antigens or antibodies to detect relevant antibodies or antigens. Alternatively, immunological based methods are limited to detect a broad range microbial cultivation, which may cause false-negative results (Fredricks and Relman, 1999); however, antigens and antibodies may combine unspecifically because of their similar structure, which is called cross-reaction (Bradshaw, *et al*, 1971; Holmberg, *et al*, 1985). The cross-reaction can cause false-positive results (Becker, *et al*, 1993; Perkins, Mirrett and Reller, 1995).

Difficult to detect unusual, unculturable or fastidious pathogens: Bacterial culture fails to yield a positive result in some instances. The reasons for a culture-negative finding in febrile patients include: (i) The causative organism may be fastidious in nature such as the HACEK group, *Brucella* spp., *Neiserria* spp., *Legionella* spp., *Nocardia* spp. and cell-wall deficient organisms. (ii) Some organisms are cell dependent such as *Coxiella burnetii, Bartonella* spp. and *Chlamydia* spp (Millar, *et al*, 2000). (iii) Some bacteria are novel or unusual, on the one hand, there are no suitable culture media to make them grow; and on the other hand, some of them can be cultured but there are no enough detection methods to identify them. For the commercial systems, there is no enough information in their databases and the databases need updating. Holmes and colleagues evaluated the biolog

system with 214 strains of nonfermenters representing 15 species and fund that even the large number of tests available may not be adequate for discriminating all clinically relevant pairs of taxa (Holmes, *et al*, 1994)

Unable to detect by culture after antibiotics used or poor sample handling: After antibiotic treatment, bacteria will be killed significantly and bacterial concentration in clinical specimens will be reduced sharply. In this situation, not only Gram stain, but also bacterial culture will be difficult to yield a positive result. For example, meningococci can be cultured from blood in 50% of untreated patients (Newcombe, *et al*, 1992), but pre-admission antibiotic treatment reduces the chance of a positive blood culture to =5% (Cartwright, *et al*, 1992). This is one of the important reasons for culture-negative infectious diseases. For some sensitive bacteria, for example, *Neisseria* spp., if the clinical specimens are not kept well in collection and transportation, the bacteria will break down quickly and die before culture.

Time consuming: This is a weak point for microbe culture. Generally speaking, most bacteria need at least 24 hours to grow for a detectable result to be obtained. For some slow growing organisms, e.g., fungi and *Mycobacterium tuberculosis*, the culture time will be up to few weeks.

MOLECULAR DETECTION METHODS

Comparing conventional detection methods which have been developing over a century, molecular detection methods are really very young which is just for a few decades. Although deoxyribonucleic acid, or DNA, was discovered in the late 1860s, it was not used until the restriction enzyme and the recombinant DNA techniques were discovered in the 1970s. During a century time, many scientists did lot of work to find the profound mystery of DNA. To trace the development of molecular detection methods, we should remember some pioneers and their discoveries.

In 1869, Johann Friedrich Miescher, a Swiss physician, discovered a weakly acidic substance of unknown function in the nuclei of human white blood cells. This substance will later be called deoxyribonucleic acid or DNA. The substance was largely ignored for nearly a century because it seemed too simple to serve any significant purpose. This view changed dramatically in 1949 Erwin Chargaff, a biochemist, reports that DNA composition is species specific; that is, that the amount of DNA and its nitrogenous bases varies from one species to another. In addition, Chargaff finds that the amount of adenine equals the amount of thymine and the amount of guanine equals the amount of cytosine in DNA from every species. During this time scientists discovered that chromosomes, which were known to carry hereditary information, consisted of DNA and proteins. In 1928, Franklin Griffith, a British medical officer, discovered that genetic information could be transferred from heat-killed bacteria cells to live ones. This phenomenon, called transformation, provides the first evidence that the genetic material is a heat-stable chemical. In 1944 Oswald Avery, a Canadian physician and bacteriologist and his colleagues Maclyn McCarty and Colin MacLeod, identified Griffith's transforming agent as DNA. Experiments conducted throughout the 1940s showed that DNA actually seemed to be the genetic material. However, it was still not known what the structure of DNA was and how such a simple molecule could contain all the information needed to produce a human being or other living organisms until 1953 James Watson and Francis Crick discover the molecular structure of DNA. After building successive scale models of possible DNA structures, they deduced that it must take the twisted-ladder shape of a double helix. The sides of the ladder consist of a backbone of sugar and phosphate molecules. The nitrogen-rich bases, A, T, G and C, form the rungs of the ladder on the inside of the helix. The pair discovered that base A would only pair with T, while G would only pair with C. This is known as complementary base pairing and neatly explains DNA's equal amounts of A and T, or G and C (Watson and Crick, 1953). They were awarded the Nobel Prize in Physiology or Medicine in 1962 for their discovery, shared with Maurice Wilkins, whose work with Rosalind Franklin on X-ray crystallography had provided crucial evidence. In 1961, François Jacob and Jacques Monod develop a theory of genetic regulatory mechanisms, showing how, on a molecular level, certain genes are activated and suppressed and they were awarded the Nobel Prize in Physiology or Medicine in 1962 for their contribution. In 1961 Marshall Nirenberg, a young biochemist at the National Institute of Arthritic and Metabolic Diseases, discovered the first triplet—a sequence of three bases of DNA that codes for one of the twenty amino acids that serve as the building blocks of proteins. Subsequently, within five years, the entire genetic code was deciphered. At end of 1960s, almost all about the DNA structures and functions were understood in theory, but people still could not get any gene as they wanted or change any gene as they needed until 1970s when some in important enzymes were discovered. The 1970s was the beginning of molecular biology blasting off. In 1970, Hamilton Smith, an American microbiologist, isolated the first restriction enzyme, an enzyme that cuts DNA at a very specific nucleotide sequence (Smith and

Wilcox, 1970). Over the next few years, several more restriction enzymes were isolated. He shared the Nobel Prize in Physiology or Medicine with Werner Arber and Daniel Nathans in 1978 for his discovery. In 1972, Paul Berg assembled the first DNA molecules that combined genes from different organisms. Results of his experiments represented crucial steps in the subsequent development of recombinant genetic engineering. (Jackson, *et al*, 1972). In 1980 Paul Berg shared the Nobel Prize in Chemistry with Walter Gilbert and Frederick Sanger, for "his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant DNA. In 1973, Stanley Cohen and Herbert Boyer combined their efforts to create the construction of functional organisms that combined and replicated genetic information from different species. Their experiments dramatically demonstrated the potential impact of DNA recombinant engineering on medicine and pharmacology, industry and agriculture (Cohen, *et al*, 1973). In 1976 Herbert Boyer confounded Genentech, the first firm founded in the United States to apply recombinant DNA technology. Walter Gilbert (with graduate student Allan M. Maxam) and Frederick Sanger, in 1977, working separately in the United States and England, developed new techniques for rapid DNA sequencing. The methods devised by Sanger and Gilbert made it possible to read the nucleotide sequence for entire genes, which run from 1,000 to 30,000 bases long. For discovering these techniques Gilbert and Sanger received the Albert Lasker Medical Research Award in 1979 and shared the Nobel Prize in Chemistry in 1980 (Genome News Network, http://www.genomenewsnetwork.org). In the 1970s, nucleic acid hybridization methods were mostly used methods and DNA probes were the powerful tools in molecular biology, microbiology, virology, genetics and forensics etc. Although hybridisation methods are highly specific for detecting targets, they are limited by their sensitivity. To get more sensitive and specific methods, Kary Mullis conceived and helped develop polymerase chain reaction (PCR), a technology for rapidly multiplying fragments of DNA in 1983 and he was awarded the Nobel Prize in Chemistry in 1993 for this achievement. In 1985, Saiki and his colleagues first used the new method to detect patient's β-globin gene for diagnosis of sickle anaemia (Saiki RK, *et al*, 1985). In 1987, Kwok and colleagues identified human immunodeficiency virus (HIV) by using PCR method (Kwok, *et al*, 1987). This is the first report the application of PCR in clinical diagnosis infectious disease.

Over the past 20 years, molecular techniques have been developed very broadly and fast. The nucleic acid amplification technology has been opened a new century for microbial detection and identification (Mullis and Faloona, 1987; Eisenstein, 1990; Nissen and Sloots, 2002). At moment, molecular detection methods, especially PCR based methods, have been becoming more and more important detection methods in clinical diagnosis laboratory. Here is a summary about the molecular detection methods used in laboratory.

Nucleic acid hybridization: Nucleic acid hybridization is based on the ability of two single nucleic acid strands that have complementary base sequences to specifically bond with each other and form a double-stranded molecule, or duplex or hybrid. The single-stranded molecules can be RNA or DNA and the resultant hybrids formed can be DNA-DNA, RNA-RNA, or DNA-RNA. Hybridisation assays require that one nucleic acid strand (the probe) originates from an organism of known identity and the other strand (the target) originates from an unknown organism to be detected or identified. The probes are capable of identifying organisms at, above and below the species level. Hybridization reactions can be done using either a solution format or solid support format. The solid support formats include: filter hybidizations, sandwich hybridizstions and in situ hybridisations. Nucleic acid hybridization methods were developed in the 1970s and they are still used in microbial detection and identification today (Murphy and Attardi, 1974; Reingold, 1999; Willems, *et al*, 2003). They are also the important detection tools in real-time PCR such as TaqMan and LightCycler (Guiver, *et al*, 2000; Kearns, *et al*, 2002; Kosters, *et al*, 2002).

Polymerase chain reaction (PCR): Principle of PCR Polymerase chain reaction (PCR) is an enzyme-driven, primer-mediated, temperature-dependent process for replicating a specific DNA sequence *in vitro* (Gray and Fedorko, 1992; Fredricks and Relman, 1999). The principle of PCR is based on the repetitive cycling of three simple reactions, the conditions of which vary only in the temperature of incubation. The three simple reactions include:

Denaturing: When temperature is around 95°C, template DNA double strand is separated to two single strands.

Annealing: When temperature drops down to 55°C, two specific oligonucleotide primers bind to the DNA template complementarily.

Extension: When temperature rises to 72°C, DNA polymerase extends the primers at the 3 terminus of each primer and synthesizes the complementary strands along 5 to 3 terminus of each template DNA using

desoxynucleotides containing in media. After extension, two single template DNA strands and two synthesized complementary DNA strands combine together forming two new double strand DNA copies.

After extension, the reaction will repeat above steps. Each copy of DNA may then serve as another template for further amplification. PCR products will be doubled in each cycle. After n cycles (30-50), the final PCR products will be 2^n copies of template DNA in theory and it just needs few hours.

Specific PCR: Specific PCR is the simplest PCR approach which is designed just for detecting some specific target microbes. In specific PCR, primers are designed complimentary to a known DNA target and specific for the microbe being assayed. This is a key point for specific PCR if the primers are strictly specific for the aimed microorganisms. Because the result is specific for detected microbe, this method can be used as direct detection and identification method. This is the most used method in diagnosis of infectious diseases. A lot of bacteria, such as *Mycobacterium tuberculosis*, *Neisseria meningitisis*, *Burkholderia* spp. can be identified by specific PCR directly (Glustein, *et al*, 1999; Saves, *et al*, 2002; Chan, *et al*, 2003).

Multiplex PCR: In multiplex PCR, two or more primer pairs are included in one reaction tube and two or more DNA templates are targeted simultaneously. This is the simple way to detect few different bacteria in one PCR reaction. In multiplex PCR, the primer pairs should be specific to the target gene and the PCR products should be in different sizes, then gel electrophoresis will provide an initial idea of which PCR reactions took place (Geha, *et al, 1994*; Hoshino, *et al*, 1998; Bindayna, *et al*, 2001).

Nested/Semi-Nested PCR: In this approach, DNA is amplified for two sets. The first PCR set will produce a bigger PCR product than that in second PCR set. The second PCR set will use the first PCR products as template DNA to amplify the DNA second time. The primers in second PCR set can be different with the first set (nested) or one of the primers is same as the first set (Semi-nested). This method can be used to increase the sensitivity of detection or to identify the first set PCR products when the primers in second PCR set are species-specific.

Broad range PCR: Broad range PCR is a very useful approach for detecting any existing microbe universally. The primers in broad range PCR are selected from the conserved regions of a particular gene that is shared by a given taxonomic group (Fredericks, *et al*, 1999). This crucial important for broad range PCR to select real broad range primes. Because16S rRNA gene is found in all bacteria and contains certain conserved regions of sequence, it has been mostly used as the broad range PCR target gene for detecting bacteria (Drancourt, *et al*, 2000; Harris, *et al*, 2002; Song, *et al*, 2003). 23S rRNA is similar with 16S rRNA gene and is also often used as broad range PCR target gene (Kotilainen, *et al*, 1998; Relman, 1998; Rantakokko-Jalava, *et al*, 2000). Because there is no so much information about 23S rRNA gene in database as 16S rRNA gene, 23S rRNA gene is not so popular as 16S rRNA gene used in broad range PCR. Another part used in broad range PCR is the inter spacer region between 16S and 23S rRNA genes. In this broad range PCR approach, the forward primer is from 16S rRNA gene and the rewords primer is form 23S rRNA gene (García-Martínez, *et al*, 1999; Hinrikson,*et al*, 1999; Xu and Côté, 2003). In broad range PCR approach for fungi, generally the 18S, 5.8S and 28S rRNA genes are selected as the target genes which have a relatively conserved nucleotide sequence among fungi and also include the variable DNA sequence areas of the intervening internal transcribed spacer (ITS) regions called ITS1 and ITS2. (Henry, *et al*, 2000; Iwen, *et al*, 2002; Xu, *et al*, 2002; Pasricha, *et al*, 2003).

Unlike specific PCR, broad range PCR amplifies DNA unspecific, so this method just can detect but cannot identify microbes directly. To identify PCR products, the PCR amplicons can be analysed by several different methods.

Nested PCR: After broad range DNA amplification, species-specific primers are used in the second set DNA amplification. The result can be detected by gel electrophoresis (Saruta, *et al*, 1997; Backman, *et al*, 1999).

DNA probe hybridization: The broad range PCR products can be identified by using species-specific DNA probe hybridization. The probe and the PCR product are incubated together in a single test tube and the binding of probe to the target is measured without further reaction (Greisen, *et al*, 1994; Dicuonzo, *et al*, 1999; McCabe, *et al*, 1999).

DNA enzyme immunoassay (DEIA): In this method, an anti-dsDNA antibody particularly recognizes the hybridization product resulting from the reaction between target DNA and a DNA probe. The final product is revealed by means of a colorimetric reaction. The DEIA increases the sensitivity of a previous PCR by including enzymatic reactions. The hybridization between specific

probe and PCR-amplified target DNA, as well as the formation of target DNA/probe hybrids and anti-dsDNA antibody complex, also enhances the specificity (Seward and Towner, 2000; Hong, *et al*, 2003).

Single-strand conformation polymorphism (PCR-SSCP): SSCP generally is used as microbe typing and mutation detection method. It also can be used as microbe identification. After PCR product is denatured to two single-stranded DNAs, the physical conformational changes in single-stranded DNA are based on the physiochemical properties of the nucleotide sequence. Conventionally, the variations in the physical conformation are detected in a nondenaturing poluacrylamide gel electrophoresis and stained with silver. Also the result can be detected by using fluorescence-labelled primers and analysis on an automated DNA sequencer (Widjojoatmodjo, *et al*, 1994; 1995).

Restriction endonuclease digestions (PCR-RFLP): After restriction endonuclease digestions, the amplified DNA fragments are cut to different small fragments according to their DNA sequences. The resulting fragments can be separated by gel electrophoresis and/or then transferred to a nylon membrane. RFLP usually is used as microbe typing and epidemiological investigation method. It s also reported to use as microbial detection method (Lu, *et al*, 2000; Sato, *et al*, 2003).

DNA sequence: DNA sequence can separate microbes at least in genus and is the mostly used microbial identification method after broad range PCR. The most targeted gene is 16S rRNA gene. In 16S rRNA gene, apart from some very conserved parts which are generally used as broad range PCR primer targets, it also has the highly variable portions which can provide unique signatures to any bacterium and useful information about relationships between them. Although 23S rRNA gene is larger than 16S rRNA gene (approximately 2.5 kb for 23S, 1.5 kb for 16S), 16S rRNA gene is still the first choice to be used because there is more information about 16S rRNA gene than that about 23S rRNA gene in database and the 16S rRNA gene may well contain enough phlogenetically informational sites to perform well as a diagnostic target (Tang, *et al*, 1998; Kolbert and Persing, 1999; Qian, *et al*, 2001).

Reverse transctiption-polemease chain reaction (RT-PCR): RT-PCR is the technique of synthesis of cDNA from RNA by reverse transcription (RT) first and amplification of a specific cDNA by polymerase chain reaction (PCR) followed. This is the most useful and sensitive technique for mRNA detection and quantitation currently available. RT-PCR is mostly used to detect virus and microbial mRNA (Shimizu, *et al*, 2002; Burtscher and Wuertz, 2003; Culp and Christensen, 2003; Habib-Bein, *et al*, 2003).

Real-Time PCR: In 1993, Higuchi first described a simple, quantitative assay for any amplifiable DNA sequence (Higuchi, *et al*, 1993). This method is based on using fluorescent labelled probes to detect, confirm and quantify the PCR products as they are being generated in real time. In recent years, some commercial automate real-time PCR systems have been available. In these systems, some of them, such as the LightCycler™ and the SmartCycler®, perform the real-time fluorescence monitoring by using fluorescent dyes such as SYBR-Green I, which binds non-specifically to double-stranded DNA generated during the PCR amplification (Kearns, *et al*, 2002; Kösters, *et al,* 2002; Spiess, *et al*, 2003). Some others, such as the TaqMan,, use florescent probes that bind specifically to amplification target sequences (Guiver, *et al*; 2000;. Corless, *et al*, 2001). At moment, some broad range primers and probes targeting the 16S rRNA gene have been developed in these systems to detect and identify bacteria universally (Corless, *et al*, 2000; Nadkarni, *et al*, 2002). The real-time PCR systems not only can reduce the detection time (results can be ready in less than on hour), but also can reduce contamination risks because amplification and detection occur within a closed system and at meanwhile, they are easy to make the quality control.

DNA sequence: In 1977, two different methods for sequencing DNA were developed: the chain termination method and the chemical degradation method. Both methods were equally popular to begin with, but, the chain termination method is improved quickly and this method is more commonly used today. This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis. The fixed laser beam excites the flurescently labelled DNA bands and the light emitted is detected by photodetectors located behind the gel. The photodetector signals are collected and sent to the computer for storage and processing.

DNA sequence is the most accurate and definitive way to identify microbes because the microbes are identified by base pair to base pair of the nucleic acid. The DNA sequences of the variable regions form the basis of phylogenetic classification of microbes (Doolittle, 1999).

By sequencing broad range PCR products, it is possible to detect DNA from almost any bacterial species. After comparing the resulting sequences with known sequences in GenBank or other databases, the identity of the bacteria enquired can be revealed. Since 1990s, 16S rDNA sequencing has been a powerful tool and more and more used in microbial detection and identification, especially for some unusual, unculturable or fastidious and slow growing pathogens or after antibiotics used (Relman, *et al*, 1990; Trotha, *et al*, 2001; Fouad, *et al*, 2002; Harris, *et al*, 2002). It is becoming a routine method of diagnosing and combating infectious diseases in diagnostic laboratory (Rantakokko-Jalava, *et al*, 2000; Harris and Hartley, 2003).

Advantage and disadvantage of molecular detection methods: Comparing with conventional detection methods, molecular methods are still very young. Although they have some unique characteristics, the methods still have some disadvantages which need to be improved.

Advantage

Sensitivity: Molecular methods are much more sensitive than conventional methods. Especially PCR based methods can be extremely sensitive. After 30 cycles, a single copy of DNA can be increased up to 10^7 copies (Forbes, *et al*, 1998) under the right conditions. In this sensitivity, clinical specimens can be detected directly but not need to be cultured first. Even sometimes the concentration of the amplified DNA is still low after first round PCR, Nested/Semi-nested PCR can increase the sensitivity much more then the DNA concentration will be high enough to be analysed.

Specificity: Although some microorganisms, especially that from the same family, are similar in morphology and even in biochemical characters, all of them have their own unique nucleic acid sequences. The target of molecular methods is just the unique nucleic acid sequences, so the methods are highly specific. Unlike immunologic methods which are based on the structures of antigens and antibodies, molecular methods are based on the complementary nucleic acids, i.e. A-T and C-G, so there is no possibility to have cross-reaction. In specific PCR, the unique oligonucleotide primers complementary to these unique segments of DNA can only amplify the microbial DNA from the organism being sought. In broad range PCR, although the universal oligonucleotide primers complementary to conserved regions of microbial DNA can amplify more diverse groups of organisms, like 16S rRNA gene broad range PCR, the PCR amplicons can still be identified by using the methods such as DNA probes and DNA sequence we mentioned above.

Table 1: The time required for PCR identification

Item	Consuming time (h)
DNA extraction	1
PCR amplification	3
Gel electrophoresis	1
Preparing sequencing reaction	1/2
Cycle sequencing	5-10
Result analysis	1
Total time	11.5-16.5

High speed: Comparing to the microbial cultivation method, the speed of molecular methods is much higher. For example, each cycle in ordinary PCR just needs few minutes (usually 3-5 min) depending on the size of amplified PCR products. For 30-40 cycles, total time may be about 2-3 h the time for sequencing needs about 5-10 h depending on the PCR amplicon size and the sequencer type. The time for preparing samples, such as DNA extraction, electrophoresis and PCR products cleaning, is about 3 h. The total time for molecular identification is about 16 h (Table 1).

The time for microbial culture at least is about 48 h. For some slow growing, or fastidious microbes, the culture time will be for days or even few weeks.

In recent years, the application of real-time PCR causes a big revolution in PCR based molecular detection. After DNA extraction, the sample is detected by the automatic system directly and the consuming time is just about 30-45 min. The total time for real-time PCR is only about 2 h, which will be very helpful for clinical doctors to manage the patients.

No limit for detecting new or atypical organisms: This is a unique point for broad range PCR. By using universal primers that are targeted at conserved regions of rDNA, broad range PCR is possible to detect DNA from almost any bacterial species. The identity of the bacterium captured is revealed by nucleotide sequencing of the PCR product followed by comparison of this sequence with known sequences located in GenBank or other databases. It is estimated that <1% of the bacteria present on earth have been described to date using cultivation technology (Fredricks and Relman, 1999). Hence, it will be not surprising that some new bacteria come up and cause some human diseases time to time. In this situation, conventional culture method will have no power to cope with, but broad range PCR can identify the novel bacteria. On the other hand, broad range PCR also can identify the bacteria which may switch certain metabolic traits on and

off, leading to confusion when trying to identify using traditional phenotypic tests, called atypical bacteria. At moment, 16S rDNA broad range PCR and sequencing is a very powerful tool and by far the single most common molecular technique used for bacterial species identification and clinical atypical bacteria identification (Reischl, *et al*, 1998; Tang, *et al*, 1998; Tortoli, *et al*, 2001; Xu, *et al*, 2002; Xu, *et al*, 2003).

No affection for pre-using antibiotics: Antibiotic admission before clinical samples were taken is an important reason for culture negative infectious diseases. Because molecular methods are based on bacterial DNA, pre-using antibiotics will not affect molecular detection. This is important not only for clinical diagnosis, but also for epidemiology. For some acute infectious, such as acute bacterial meningitis, early antibiotics treatment can largely reduce mortality. At mean time, the culture positive rate is reduced sharply as well. This is not only difficult to get an accurate diagnosis, but also difficult to get correct epidemic information. Molecular methods can give all the information quickly and accurately, no matter how early and how much antibiotics were used.

Disadvantage

False positive: The false positive is a common problem in PCR based molecular methods, especially in broad range PCR. The main reason of false positive is from contamination. Indeed, contamination is the biggest enemy for PCR based molecular detection, especially for broad range PCR. It is also a big barrier for PCR techniques to be used in routine test in clinical diagnostic laboratories. The contamination may come from many different sources such as clinical specimens, PCR reagents, sample preparing, PCR setting up and post PCR. Because the PCR methods are very sensitive, even a little contamination, especially the post PCR products contamination, can cause the false positive results. The more sensitive of the PCR methods, the higher risk of the contaminations. To eliminate contamination and keep PCR sensitivity in a high level, people tried to use different methods such as to separate pre-PCR and post PCR rooms completely, to select high quality reagents, to irradiate PCR reagents with UV light, to avoid cross contamination in sample preparation and PCR setting up, etc. (Fredricks and Relman, 1999; Corless, *et al*, 2000; Millar, *et al*, 2002; Harris, *et al*, 2003). Without reducing contamination deeply, it is difficult to move PCR methods to diagnostic laboratory as routine test methods.

In specific PCR and DNA probe hybridization, there is another reason to get false positive results: unspecific primers. If the primer pair is not highly specific to the detected organisms, it will pick up some other similar organisms as well. Then false positive will happen (Trinker, *et al*, 1996; Borrow, *et al*, To 1998). This problem is not so difficult to solve as contamination. To design highly specific primers, people should select the highly stable and specific target gene first and then align the related organism sequences as many as possible and also, try to amplify different organisms as many as possible before using the new specific primers.

False negative: False negative is another common problem in PCR based methods. There are a few reasons may cause PCR false negative. The most common reason is that the samples may contain PCR inhibitors that interfere with DNA amplification. The inhibitors can be different substances from clinical samples or from reagents. For example, as PCR inhibitors, heme is from blood sample; EDTA is from PCR reagents; sodium polyanetholsulfonate (SPS) is from blood culture media. To remove the inhibitors, different methods can be used. One way is diluting the inhibitors; another way is using different DNA extraction methods. For instance, to remove the very strong PCR inhibitor SPS, Fredricks and Relman used dilution and benzyl alcohol extraction protocol (Fredricks and Relman 1998); Kulski and Pryce, Millar and colleagues used alkali wash and heat lysis method (Kulski and Pryce 1996; Millar, *et al*, 2002); Qian and colleagues added 0.5% bovine serum albumin to blood culture samples (Qian, *et al*, 2001;).

Another reason for false negative result may be that the concentration of template DNA is too low and the sensitivity of PCR amplification is not high enough to detect it. To solve this problem, one way is to concentrate the DNA sample; another way is to increase the PCR sensitivity, for example, to use nested/semi-nested PCR.

Limit detection range by specific PCR and probe-based detection: Specific PCR and DNA probe-based methods are highly sensitive and specific to the microbes detected. The problem is that these methods only can detect the expected microbes. Although people tried to use multiplex PCR and DNA probes to detect several common pathogens simultaneously (Greisen, *et al*, 1994; Radstrom, *et al*, 1994; Saruta, *et al*, 1997), they still just could detect the pathogens they were looking for. If the causative organism is not common bacterium, but some unusual one, they will be failed to get positive results. Then it will be culture negative and PCR negative either infectious disease. In this case, broad range PCR will be very helpful.

Difficult to keep quality control: Over the last two decades, molecular methods have been accepted by clinical diagnosis laboratory and becoming more and more popular. Some people even tried to develop the molecular methods to routine service. The big problem at moment is difficult to keep the quality control. Most laboratories are using numerous in-house PCR assays. The performances reported, both for the sensitivity and the specificity of these assays are very divergent. (Bretagne, 2003). There are no any standard to follow.

Unable to detect antibiotics sensitivity of pathogens: Although PCR assays can detect antibiotic resistance genes in microbes, for example, the methicillin resistance gene (*mecA*) in *Staphylococcus aureus*, the vancomycin resistance genes (*vanA, vanB and vanC*) in vancomycin-resistant entreococci, molecular methods cannot provide data about antibiotic susceptibility of organisms which just performed in living microbes. Sometimes it is very important for clinical doctors to get information for antibiotics treatment.

High cost: Because molecular methods need high technology, expensive equipment and reagents, the costs will be higher than that in other detection methods, such as microbial culture. As mentioned before, it is said that to detect one sample, culture method needs about 2-3 pounds, but PCR and sequencing method needs about 10 pounds.

CONCLUSIONS

Over one hundred years developing, conventional methods have been stable, reliable and mature. Although the conventional methods have some week points as we mentioned above, the methods, especially microbial culture, the golden standard diagnosis method, have played a key important role in the diagnosis of infectious diseases since the methods started to use and they will still keep the same role for a quite long period in the future until molecular methods are developed good enough to take place the conventional methods. Even when molecular methods become a key role in the future, conventional methods will be still an important tool to know the morphology and physiology about the bacteria and their virulence, antibiotic susceptibility.

Molecular methods will be the direction for development of infectious diagnosis. Although molecular methods have some limitations to use as routine service right now, the advantage methods, especially PCR-sequence based methods, will be dominative methods in laboratory diagnosis of in the near future, because of their high seep, high sensitivity and high specificity.

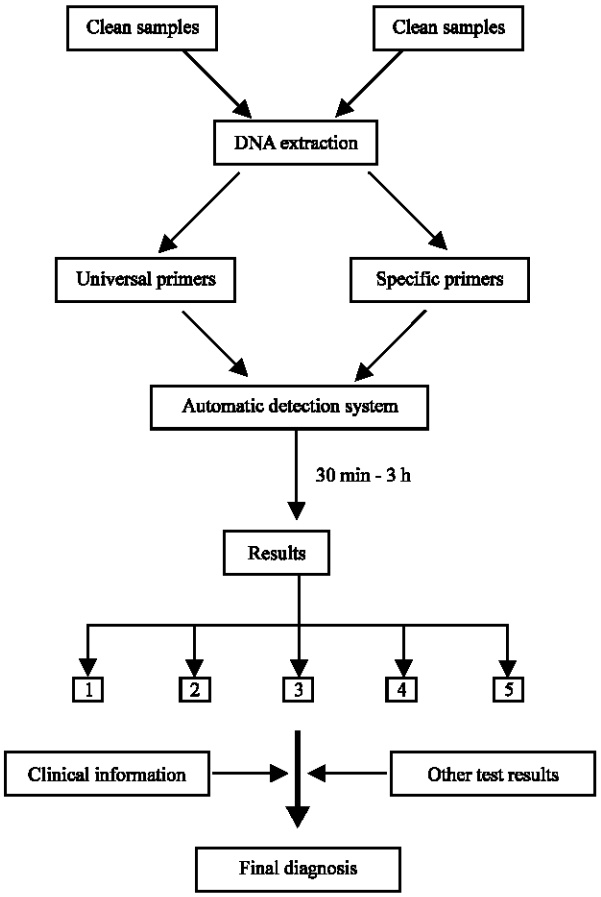

Fig. 2: Molecular diagnosis workflow

In the future, all molecular methods will have quality standards and all work will be done in automatic detection systems. The automatic systems will have the ability to detect all existing microbes simultaneously and list them in order according their DNA concentration. The clean clinical specimens such as cerebrospinal fluid (CSF), blood and some other body fluid, will be detected by using broad range primers; and the unclean clinical specimens will be detected with species-specific primers. Clinical doctors will combine the laboratory detection results with patients' clinical information to make the final diagnosis and decide how to treat the patients. The workflow is showed as Fig. 2.

At mean time, all samples also will be detected with conventional methods to confirm the results from molecular methods and provide antibiotic sensitivity results to clinical doctor to guide the antibiotics treatment.

To reach above aims, there is still long way to go. Lot of essential work should be done first. The work includes optimising the work conditions, such as DNA extraction, PCR primers, eliminating contamination, establishing efficient quality control systems and automatic detection

1systems, adding more high quality sequence information to GenBank or other databases. Conventional methods still should be main detection methods and molecular methods can be complementary methods to help doctors make fast and right diagnosis. The in- house methods are the way to accumulate all the information. Once we get enough information, it will be easy to get all the work under good control step by step.

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