Laboratory Test for Diagnosis of Parasitic Diseases

Jinli Lou, Yanhua Yu, and Fangfang Dai

Parasitic diseases generally develop chronically, and most patients experience non-specific symptoms and show unobvious physical signs. Except medical history and physical examination, the diagnosis is mainly based on the laboratory tests. The commonly applied laboratory tests for the diagnosis of parasitic diseases include etiological examination, immunological assay and molecular biological examination.

Etiological examination is to detect the parasitic pathogen from such specimens as stool, blood, bone marrow, sputum, body excretions and secretions as well as tissue from patient. It can be categorized into staining test and non-staining test. The etiological examination by staining is commonly applied to detect larvae, eggs, and cysts of parasite, while the examination with no staining is selected to detect parasite from blood and tissue fluid. Etiological examination is the most reliable way for a definitive diagnose of parasitic infection or disease.

Immunological assay serves as a convenient diagnostic examination of parasitic infection. With the development and improvement of technology in immunological antigen detection, its clinical value in diagnosis has been increasing and the technique has been applied in epidemiological studies and surveillance of epidemics. The conventional examinations include intracutaneous test, circunoval precipitin test, cercarien hullen reaction and indirect haemagglutination test. Nowadays, fluorescence immunoassay, enzyme immunoassay and immunoblotting have been gradually applied to the laboratory diagnosis of parasitic diseases. Therefore, the diagnostic sensitivity and specificity of parasitic diseases have been greatly improved.

In recent years, molecular biology for diagnosis of parasitic diseases has gained rapid development. By using the technologies of DNA probe, PCR and DNA sequencing, DNA fragments and sequence of parasite can be detected in specimens, which have been applied in laboratory diagnosis of trypanosomosis, leishmaniasis, pneumocystosis and toxoplasmosis. Via high throughput and automated DNA hybridization or immunoassay, the biochip technology can be applied to simultaneously examine multiple specific target molecules in one biochip. This will bring about a revolution to high throughput combined detection of infectious diseases and genetic disorders.

In this chapter, we will briefly introduce the laboratory examinations and their clinical application. In combination to the medical history of patients and their clinical symptoms, these laboratory examinations facilitate the diagnosis of parasitic diseases. In addition, they are significance in assessing therapeutic efficacy and predicting prognosis.

6.1 Virological Diagnosis

The virus is the simplest and smallest microorganism whose infection is very common. About 70–80 % of infectious diseases are caused by viruses. Up to now, it has been proved that more than 500 species of virus are pathogenic to humans and some are extremely dangerous, such as viruses inducing avain influenza or SARS. Therefore, virological diagnosis is of great significance in controlling the spread of virus as well as diagnosing and preventing corresponding diseases.

The general principles in laboratory diagnosis of viral diseases are specific, sensitive, rapid, and simple. Based on the epidemiological and clinical features, the suspected pathogenic virus should be primarily judged. And the corresponding laboratory examination should be then chosen based on the biological properties of the suspected virus, immune responses of the patients, clinical course, and the condition of patients. Currently, the laboratory diagnosis of viral infection mainly relies on the conventional examinations and molecular biological examinations. The conventional examinations include virus isolation and culture, identification and serological test, while the molecular bio-

J. Lou (✉) • Y. Yu • F. Dai
Beijing Youan Hospital, Capital Medical University, Beijing, China
e-mail: 13718641405@163.com

logical examinations include nucleic acid hybridization, PCR and modern immunoassays.

6.1.1 Specimen Collection and Transport

6.1.1.1 Specimen Collection

Sampling Time
Sampling should be performed preferably in the early stage and acute stage after onset of the disease or on the first day of hospitalization. It should be performed as early as possible, preferably before treatment. In the later stages of disease, immunity may be induced in patient with less or no virus detected to adversely affect the diagnosis and treatment.

Selection of the Type of Specimen
According to the clinical symptoms and epidemiological data, the infected virus should be primarily judged and the site for specimen collection as well as the type of specimen should be correspondingly selected. The biological properties of the suspected virus should be considered when handling the specimen. For the following diseases, the virus should be isolated for laboratory diagnosis: heart disease, central nervous system infection, congenital or neonatal infection, gastrointestinal disease and respiratory infection.

Commonly Applied Ways for Specimen Collection
For possible respiratory tract infection, nasopharyngeal lavage liquid or sputum is commonly collected for laboratory diagnosis; faeces specimen for possible intestinal infection; aseptic collection of cerebrospinal fluid for possible brain infection; effusion in herpes for possible skin rash; and blood for the cases of viremia. The collected specimen should contain infected cells. The specimen for serological test, especially those for detection of IgG antibody against pathogen, should be collected at the early and convalescent stages for double sera, followed by analyzing dynamic changes of the double sera antibody titer. Only when the serum antibody titer at the convalescent stage is at least four times as high as that in the early stage, the diagnosis can be defined.

6.1.1.2 Transport and Preservation of Specimen
After the collection, the specimen should be maintained aseptic in freezing and moisturized environment and sent for examination immediately. It is more favorable to deliver the specimen into the laboratory within 1–2 h after its collection. Serum specimen should be labeled and preserved at a temperature of −20 °C. Virus tends to be inactivated at room temperature, and the specimen should be maintained at a low temperature and delivered for examination immediately. If transport is needed, the specimen should be stored in ther-

mos flask filled with ice or low temperature materials. And the tissue specimen should be stored at a low temperature with 50 % glycerol buffered saline containing antibiotics or DMSO. If the tissue specimen cannot be delivered immediately, it should be stored at a temperature of −70 °C.

6.1.2 Virus Isolation and Identification

The ways for virus isolation, culture and identification are complex, which require specific conditions and a comparatively long period of time, therefore, fail to be widely applied in clinical diagnosis. For the following conditions, virus isolation, culture and identification can be applied: (1) The patient shows a long-term illness course with a suspected diagnosis of viral infection, all laboratory tests for virus show negative, and isolation of the virus will provide valuable guidance for the diagnosis and treatment. (2) Emergent virus infection is suspected, or re-emergence of eliminated virus is suspected. (3) The same symptoms are suspected to be caused by different viruses. The virus isolation can be applied to define the virus pathogen. (4) It should be defined whether attenuated live vaccine used for survallience shows pathogenic mutant strain. Virus has strict intracellular parasitism. Therefore, sensitive cells, chicken embryos and sensitive animals should be selected based on different virus receptor for isolation, culture and identification of virus.

6.1.2.1 Virus Isolation and Culture

Cell Culture
Cell culture is the most commonly applied way for virus isolation. Appropriate cells should be selected based on the cytotropism of virus. The commonly used cells for virus isolation include: (1) Primary cultural cells, such as simian nephrocytes or human embryonic nephrocytes, are highly sensitive but difficult to obtain. (2) Diploid cell strain, being limited within about 50 generations, can be applied for laboratory use. But the cells may be subjected to aging and death after several generations. (3) Continuous or infinite cell line or strain, such as Hela or Hep-2 cell, can be conveniently preserved in laboratory and shows stability to virus infection, which has gained widespread application. After inoculation, the cytolytic virus can induce cytopathic effect (CPE), while the cells stably infected by virus do not show obvious CPE. However, the membrane surface of a cell infected by virus may show markers of virus expressed protein, such as hemagglutinin and specific antigen of the virus. Hatest or immunological assays may be applied to examine proliferation of the virus. Negative findings in CPE or other laboratory tests may be induced by small quantity of viruses in the specimen that is undetectable even if proliferation of the

virus does occur. After blind passage for 3 generations, negative finding definitively indicates no virus in the specimen.

Chicken Embryo Culture & Animal Inoculation of Influenza Virus

Although canine kidney cells can be selected, chicken embryonic inoculation is still the most widely applied way with sensitivity and specificity. Hemagglutination test and hemagglutination-inhibition test may be applied for further identification. Animal inoculation is the most primitive way for virus isolation, but rarely applied nowadays. Mice intracerebral inoculation is still applied for isolation and identification of rabies virus or encephalitis B virus.

6.1.2.2   Virus Identification

Indices for Virus Proliferation in Cultured Cells

Cytopathic Effect (CPE)

Most of the viruses are cytolytic, whose proliferation in sensitive cells causes CPE. CPE may show increased particles within infected cells that cluster or fuse, sometimes with formation of inclusion body. The infected cells are finally subject to cytolysis, detachment and death. Different viruses show different CPE. For instances, adenovirus can cause rounded shrinkage and clustering of the infected cells, which typically shows the appearance of grapes cluster; poliovirus can cause rounded shrinkage, scattering, necrosis and detachment of the infected cells; respiratory syncytial virus may cause fusion of the infected cells to form multinuclear giant cells. Therefore, the infected virus can preliminarily suspected based on the type of selected cell and CPE features. The enveloped virus, such as influenza virus, releases progeny viruses via budding and shows stable infection with no or only undetectable CPE. Such viruses should be identified by other examinations.

Hemadsorption

The envelope of influenza virus carries hemagglutinin. After infection of influenza virus, hemagglutinin can be found on the membrane surface of sensitive cells with infection to render their binding with erythrocytes, which is known as hemaadsorption phenomenon and is an indirect indicator in detecting orthomyxovirus and paramyxovirus.

Determination of Virus Infectivity and Virus Load

Hemagglutination Test

After the virus containing hemagglutinin is inoculated into chicken embryo or infects cells, chicken embryonic amniotic fluid and allantoic fluid or cell culture fluid should be collected, followed by addition of animal erythrocytes. For the cases with virus proliferation and extracellular release, erythrocytic agglutination occurs, which is an indicator of virus proliferation. The virus suspension can be diluted into different levels, the highest dilution degree of hemagglutination may act as a hemagglutination titer for semi-quantitative detection of virus load.

Neutralization Test (NT)

The serum containing specific antibody against a known virus should be firstly mixed with the detected virus suspension, followed by inoculating sensitive cells after a certain period at an appropriate temperature. After culture, the absence of CPE or hemadsorption indicates neutralization of the specific antibody with the corresponding virus infectivity, which is a more reliable diagnostic examination for virus infection. Different concentrations of serum containing specific antibody can be used for the neutralization test to semi-quantitatively examine the detected virus suspension based on the antibody titer.

Plaque Formation Test

Plaque formation test is a way to examine the quantity of virus in the specimen. After a certain amount of appropriately diluted virus suspension is inoculated into sensitive mono-layer cells for culture of a certain period, the cells are then covered by a layer of melting but un-concreting AGAR for continued culture. The proliferation of a single virus can dissolve and detach the mono-layer cells with infection to form a plaque that can be observed by naked eyes. One plaque is induced by proliferation of one virus. The quantity of plaques, therefore, indicates the virus load in the specimen. The final result of plaque formation test is usually indicated by plaque formation unit (PFU) per ml virus suspension, (PFU/ml).

Detection of 50 % Tissue Culture Infectious Does (TCID50)

The detected virus suspension is firstly diluted for a series of ten times, followed by inoculation of mono-layer cells, separately. After culture, the indicators such as CPE should be observed. With the virus load with the highest dilution level that can infect cells as the terminal point, the value of TCID50 can be calculated. TCID50 detection is applied to assess the virus infectivity and the virulence via assessment of CPE.

Detection of Multiplocity of Infection (MOI)

MOI primarily refers to the average of bacteriophages infected by a single bacterial cell in a specific test. But currently it is used as a quantitative indicator of the virus infectivity.

6.1.3   Rapid Diagnosis of Virus Infection

Rapid diagnosis mainly depends on non-culture identification of the virus instead of isolation and identification of the

virus. The examinations for rapid diagnosis include direct observation of viral particles in the specimen under an electron microscope or direct detection of the viral components such as antigen and nucleic acid and specific antibody of IgM.

6.1.3.1 Morphological Examination

Electron Microscopy and Immuno-Electron Microscopy

The specimen containing high concentration of viral particles ($\geq 10^7$ particles/ml) can be directly observed under an electron microscope. The specimen containing low concentration of viral particles can be observed after the viral particles are gathered by immune-electron microscopy. Otherwise, the specimen sediments after ultracentrifugation can be observed under an electron microscope to improve the detection rate. Morphological properties of the virus as well as its size and quantity can be observed and determined by electron microscopy.

Light Microscopy

Pathological specimen or specimen containing cast-off cells and needle aspiration cells may show basophilic or eosinophilic inclusions at the sites of virus proliferation such as nucleus and cytoplasm. And the inclusions has a certain value for the diagnosis of virus infection. A diagnosis can also be made according to the pathological properties of the specimen in combination to immunohistochemical staining.

6.1.3.2 Viral Protein Antigen Detection

Immunoassays are commonly applied for direct detection of the virus antigen and thus early diagnosis. Currently, the commonly used immunoassays include enzyme linked immunosorbent assay (ELISA), immunofluorescence assay (IFA) and radioimmunoassay (RIA). These immunoassays are simple in operational procedures but show high specificity and sensitivity. By using high-quality labeling of specific antibody, especially monoclonal antibody labeling, the antigen or hapten at the level of ng (10^{-9} g) to pg (10^{-12} g) can be detected. Due to the possible radioactive pollution induced by radioactive nuclide, the radioactive immunological markers are gradually replaced by non-radioactive markers, such as digoxin. Positive finding of the virus antigen in Western-blot (WB) test can define the diagnosis of virus infection.

6.1.3.3 Early Antibody Detection

Detection of Specific Antibody IgM

To detect specific antibody IgM produced after virus infection can facilitate an early diagnosis of virus infection. For instance, an early diagnosis of fetal congenital infection induced by certain virus can be made when the specific antibody IgM is detected in amniotic fluid from the pregnant woman. HBc antibody is produced early after virus infection, and the detection of specific antibody IgM against HBc is thus used as an indicator of acute hepatitis B virus infection. Detection of specific antibody IgM facilitates the early diagnosis of virus infection, but the production of specific antibody IgM shows obvious individual differences.

Western-Blot Test (WB)

The diagnosis of certain virus infection should be cautious. For instances, the initial screening test positive cases of AIDS and adult leukemia should be confirmatively diagnosed by WB test. In WB test, purified HIV is processed, followed by separation of virus protein based on their molecular weight via polyacrylamide gel electrophoresis. The virus protein is then electro-transferred to nitrocellulose membrane for preparation of blot strip, whose positive reaction to the serum from patient can confirm the diagnosis. If the serum contains antibody against an antigen of HIV, the antibody binds to the corresponding site on the virus protein blotted strip.

6.1.3.4 Detection of Virus Nucleic Acid

Most of the viral genes have been successfully cloned for the whole genome sequencing, which laid a foundation for detection of virus nucleic acid. And the detection of virus nucleic acid is another rapid and specific examination for the diagnosis of virus infection.

Nucleic Acid Electrophoresis

The nucleic acid of orthomyxoviridae and reovirus genus is segmental. For instances, the influenza A and influenza B virues have 8 segments of nucleic acid; the influenza C virus 7 segments; reovirus 10 segments; rotavirus 11 segments. The nucleic acid of rotavirus can be directly extracted from the specimen, and the 11 segments can be shown without endonuclease hydrolysis. After polyacrylamide gel electrophoresis and silver staining, 11 strips can be observed clearly on the gel plate. In combination with clinical manifestations, the diagnosis can be made.

Nucleic Acid Hybridization

Nucleic acid hybridization is performed by using a single-stranded nucleic acid with known sequence as a probe, which should be labeled in advance by a radioactive nuclide (such as ^{32}P or ^{131}I) or biotin, digoxin, horse radish peroxidase. Under certain conditions, the probe binds to target sequence in the specimen according to the complementary base pairing. By examining the marker, the existence of specific virus nucleic acid sequence is proved for etiological diagnosis of virus infection. The commonly used nucleic acid hybridization techniques are as follows:

Dot Blot Hybridization

The DNA or RNA for examination is dotted on the hybridation membrane. After deformation, it hybrids with the labeled probe. Radionuclide or non-radioactive marker can be detected by autoradiography or enzyme reaction.

In Site Hybridization

It is a special examination combing nucleic acid hybridization with cytology. On the pathological section, the DNA or RNA released by cells in situ hybrids with labeled probe with specific nucleic acid. After staining, the intracellular distribution of examined virus nucleic acid and its relationship with cell chromosomes can be directly observed.

Southern Blot and Northern Blot Hybridization

The DNA or RNA extracted from the specimen is cut by using restriction endonuclease, followed by agarose electrophoresis to form endonuclease strips, which are then transferred onto nitrocellulose membrane to hybrid with the labeled probe sequence. In such a way, specific sequence of virus DNA or RNA can be detected.

Polymerase Chain Reaction (PCR)

Specific and conservative virus fragments were selected as target gene. Under the effects of polymerase (Taq enzyme), the designed specific primer sequence is used to amplify the specific sequence of the virus. In such a way, virus infection can be diagnosed. Otherwise, the susceptible area of virus is selected, in combination to restrictive fragment length polymorphism (RFLP) analysis or sequencing, for studies on virus typing and mutation. By using reverse transcription PCR (RT-PCR), the RNA virus is detected. And along with increased demands for laboratory diagnosis, the PCR technology has been gaining development, with emergence of ligase chain reaction (LCR) and real-time PCR in recent years. These new techniques integrate gene amplification, molecular hybridization and photochemistry, which can be applied for real-time dynamic quantitative detection of the PCR amplification products.

Gene Chip

The integration of harvested biological information about virus gene sequencing into automation technology produces gene chip technology. Specifically, gene chip technology derives from combination of single nucleotide polymorphisms labeling with automated chain trace analysis. Its principle is to arrange a biological molecular probe or gene probe with known sequence on a tiny silicon chip or other carrier in a large scale or orderly. The gene chip is then reacted to the biological molecular sequence or gene sequence in the examined specimen. Under sequential excitation by laser, the produced fluorescence spectrum signal is collected by a receiver. And the result can be obtained after data calculating, analyzing and processing. In such a way, a large quantity of specimens can be detected simultaneously for DNA sequencing, which has prospective widespread application in the etiological diagnosis and epidemiological studies of virus infection.

Gene Sequencing

Gene sequenceing includes whole gene sequencing of the virus and characteristic gene fragment sequencing of the virus. At present, the whole gene sequencing of the known pathogenic viruses has almost been completed. The virus gene sequences in the gene pool has laid a solid foundation for the genetic diagnosis of virus infection.

6.2 Bacteriological Diagnosis

6.2.1 Specimen Collecting and Processing

The quality of specimen directly affects the validity of the laboratory test, and inappropriate collection of specimen can lead to false-negative or false-positive result. Therefore, standardized operations for quality control are necessary in each procedure including collecting, transporting and preserving the specimen to guarantee the accuracy and reliability of the laboratory test results.

6.2.1.1 General Principles of Specimen Collection

Early Collection

The specimen should be collected at the early or acute stage of the disease, otherwise during the stage with typical symptoms. In addition, the specimen should be collected before the use of antibacterial agents.

Aseptic Collection

The specimen should be protected from exogenous contamination. During the collection of such specimens from sterile parts as blood, cerebrospinal fluid, pleural effusion and joint fluid, local skin and its surrounding area should be sterilized and the procedure should be performed according to aseptic operations. During the collection of specimens from cavity or canal with opening to the external environment, such as the sinus, the fudus should be chosen for collecting tissue specimen instead of the orifice to prevent contamination from the bacterial colonies at the skin surface. Otherwise, misdiagnosis may occur. During the collection of specimens from the sites where bacteria colonies commonly exist (e.g. oral cavity), the target bacteria should be firstly determined, and the culture mediums should be specifically chosen for pathogenic culture and isolation. The collected specimen should be placed in an aseptic container after it has been

physically sterilized such as autoclaving, boiling and dry-heating. Otherwise, the specimen should be placed in a disposable sterile container which should not be processed with disinfectant or acid solution.

Choosing Different Ways Based on the Properties of the Target Bacteria

Different examinations should be applied for the collection of anaerobic bacteria, aerobic bacteria, facultative anaerobe and L-shaped bacteria. As for a urine specimen, when anaerobic infection is suspected, a sterile syringe can be used for suprapubic bladder puncture for specimen collection; when aerobic or facultative anaerobic infection is suspected, natural catheterization or clean-catch midstream urine can be chosen for its collection.

Specimen Collection for an Appropriate Quantity

The quantity of collected specimen should appropriate with representativeness. Meanwhile, some specimens should be collected from different sites at different time points. For instance, for patients with enteric fever, blood specimen should be collected in the first week after the onset; while stool specimen in the second week and urine specimen in the third week. Otherwise, the detection rate of the pathogenic bacteria can be affected.

Safe Collection

During collection, the specimens should be prevented from contamination by bacterial colonies on skin and mucosa. Meanwhile, the specimen collectors should pay attention to safety issues to prevent the spread of bacteria and to avoid being infected.

All the specimens should be collected according to the operational procedures for quality control. Clinical microbiology lab should refuse receiving specimens that fail to meet the requirements.

6.2.1.2   Specimen Processing

As for bacteria that are sensitive to the environment, such as neisseria meningitidis, neisseria gonorrhoeae and haemophilus influenzae, the specimens should be preserved at a certain temperature and immediately sent for examination. Other specimens should be sent to the lab within 2 h after collection. If immediate delivery is possible, the specimen should be preserved under a certain circumstance. For instances, such specimens as autopsy tissue, bronchial lavage, pericardial fluid, sputum and urine should be preserved at a temperature of 4 °C, while such specimens as cerebrospinal fluid and synovial fluid should be preserved at a temperature of 25 °C. Generally, the specimens for bacterial culture should not be preserved for above 24 h.

As the specimens from patients may contain a large quantity of pathogenic bacteria, safety and protection are always important regardless of the transporting distance. The opening and external surface of the container with specimen should not be contaminated by the specimen. The container should be well packed to avoid being tipped or spilt during the transport. As for the specimens of suspected fulminating infectious diseases, they must be packed according to the requirements and transported by specialized staff.

Anaerobic specimen should be placed in special bottles or tubes for transport, and sometimes it can be transported within the syringe that is used for tissue aspiration.

6.2.2   Morphological Examination of Bacteria

Morphological examination, as one of the most important bacteriological examinations, includes examinations of unstained specimens and stained specimens. Microscope is a necessary tool for the morphological observation of the bacteria.

Microscopy facilitates rapid understandings about presence of bacteria in the specimen and approximate quantity of the bacteria. In addition, the microscopic observation of the shape, structure and characteristic staining of bacteria is helpful for the preliminary identification and classification of the pathogenic bacteria, which also provides basis for further biochemical reaction and serological identification. As for certain types of bacteria, such as the acid-fast bacilli in sputum and neisseria meningitidis in cerebrospinal fluid, a preliminary diagnosis can be made based on the morphological examination, which is also an important reference for early clinical diagnosis and following treatment.

6.2.2.1   Unstained Specimen

Direct microscopic examination of unstained bacteria is mainly applied for studying the motility and mobility of the living bacteria, which includes pressure drop and hanging drop for observation under a common light microscope or dark-field microscope. Bacteria with motility can be observed moving from one site to another with obvious direction. However, bacteria without motility are shown in Brownian movement due to the push of water molecules, namely vibrating at the same location.

In clinical practice, certain pathogenic bacteria can be preliminarily identified via motility examination of the unstained specimen. For instance, for patient with a suspected diagnosis of cholera, the watery stool specimen should be collected and prepared into hanging drop or pressure drop for observation of the bacterial motility under a high power microscope or a dark field microscope. If meteorites like bacteria are observed moving back and forth, the same procedure should be repeated to prepare another hanging drop or pressure drop, followed by addition of serum containing O_1 group of vibrio cholerae for further diagnosis.

If the active motility of bacteria terminates (immobilization test positive), O_1 group of vibrio cholerae infection can be preliminarily diagnosed. In addition to the bacteria specimens, due to the difficulties in staining and the morphological characteristics, spirochetes can be prepared into unstained specimen for observation under a dark-field microscopes.

6.2.2.2 Stained Specimen

After being stained, the specimen for bacteriological examination can be observed for the shape, size and arrangement of the bacteria. In addition, the bacteria can also be classified based on their response to staining. Therefore, staining for examination is one of the most widely-used way for and plays a very important role in bacterial identification. The clinically common staining for bacterial examination includes Gram staining, acid-fast staining, fluorescence staining and special staining.

Gram Staining

Gram staining is the most classical and frequently applied staining for bacteriological examination. Except for extremely rare types of specimens such as blood, most of the specimens undergo Gram staining and microscopic examination before bacteriological culture and isolation. Based on Gram staining, the bacteria is categorized into Gram positive and Gram negative, which facilitates preliminary identification of the bacteria. In combination to characteristic morphology and arrangement of the bacteria, the pathogens can even be preliminarily identified in some cases. For instances, in patients with cerebrospinal meningitis, the cerebrospinal fluid can be collected for smear, Gram staining and microscopy. If intracellular or extracellular Gram negative diplococcocci with kidney liked shape and concave surface parallel to each other are detected, the finding of "Gram negative diplococcocci like neisseria meningitidis" can be reported. If Gram positive diplococcocci with clear surrounding capsules are detected, the finding of "Gram positive diplococcocci like streptococcus pneumoniae" can be reported. The finding after Gram staining and microscopy can provide basis for early clinical diagnosis and the following treatment.

In addition to bacterial identification, the finding by Gram staining can provide reference for clinical medication and facilitate therapeutic planning. This is because the Gram positive and Gram negative bacteria show different sensitivity to certain antibacterial agents, and their pathogenic substances (with the former generates exotoxin, while the latter generates endotoxin) and mechanisms are different.

Acid-Fast Staining

The acid-fast staining facilitates identifying acid-fast bacteria from non-acid-fast bacteria. Since most pathogens are clinically identified as non-acid-fast bacteria, acid-fast staining is not a routine bacteriological examination in clinical practice but only targets on suspected cases of such diseases as tuberculosis and lepriasis. The specimen from suspected cases of mycobacterium tuberculosis can be examined by acid-fast staining and following oil immersion lens for preliminary diagnosis. Based on the result of acid fast staining, "detected or un-detected acid-fast bacteria" can be reported, which provides important reference for clinical diagnosis and treatment.

Fluorescent Staining

With high sensitivity and efficiency, fluorescent staining provides results that can be conveniently observed and has great practical value in clinical bacteriological identification. It is mainly applied to diagnose infections of mycobacterium tuberculosis, mycobacterium leprae, corynebacterium diphtheria, shigella dysenteriae, and spirochete.

Special Staining

To observe the special bacterial structure for further identification, special staining is selected, which includes capsule staining, ink staining, spore staining, metachromatic staining and flagella staining. For instances, the specimen from suspected cases of cryptococcus neoformans infection should be performed ink negative staining. On the black background, white shining capsule of the bacteria can be observed if cryptococcus neoformans is infected. The specimen from suspected cases of corynebacterium diphtheria infection should be performed smear for examination. Based on the finding of Gram positive corynebacteria, the specimen should be further microscopically examined after metachromatic staining. If metachromatic granules are detected, the finding of "corynebacterium diphtheria like bacteria" can be reported, which provides basis for the early clinical diagnosis.

6.2.3 Bacteriological Culture and Identification

In most cases, the etiological diagnosis of bacterial infection can only be defined based on bacteriological culture and identification. Therefore, bacterial culture plays an important role in the diagnosis, prevention and treatment of bacterial infections.

6.2.3.1 Bacteriological Culture

After being transported to the bacteriological laboratory, the specimen should be immediately inoculated to an appropriate culture medium for bacterial isolation. Based on the source of specimen and the suspected pathogenic bacteria, an culture medium should be appropriately selected. For instances, blood plate, Chinese blue/MacConkey or chocolate

plate is always selected for the bacteriological culture of sputum. Specifically, blood plate is selected for culture of streptococcus pneumoniae and corynebacterium diphtheria; the Chinese blue/MacConkey plate for screening of Gram negative bacillus; chocolate plate with bacitracin for screening of haemophilus. Appropriate use of culture medium can increase the positive rate of bacteriological culture.

For such liquid specimens as blood, pleural effusion, ascites, cerebrospinal fluid, bile and pus, blood culture bottle can be selected for automated blood culture.

6.2.3.2 Identification of Pathogenic Bacteria

The suspected pathogenic bacteria that shows positive to bacteriological culture should be further identified. The mechanism underlying the bacterial identification is that different bacteria produce different metabolites due to their respective unique enzyme system with variance in substrate decomposition. Based on their own unique biochemical properties of these metabolites, biochemical examinations are applied to identify the pathogenic bacteria. In clinical practice, a preliminary bacterial identification is performed based on bacteriological morphology, staining and culture. However, most isolated unknown bacteria are identified by manual or automated operations of biochemical examination. Currently, the isolated bacteria commonly undergo strain identification and drug sensitivity test based on automated operations of biochemical reaction test. However, nowadays, mass spectrometry identification system has been applied for bacterial identification via preparation, isolation and gas ion detection. The results have demonstrated favorable consistency with those by routine biochemical examinations, and high accuracy.

6.2.4 Non-culture Bacterial Identification

In addition to microscopic examination of smears and direct bacteriological culture, non-culture bacteriological examination is also applied in the etiological identification of bacterial infections. Such non-culture examinations include immunoassay, molecular biological examination, bacteriotoxin detection and animal experiment, which can result in the etiological diagnosis in combination of the clinical manifestations.

6.2.4.1 Immunoassay

Based on the immunological principles, a known antibody is used to detect an unknown antigen or a known antigen is used to detect an unknown antibody, which is an important way for clinical diagnosis of bacterial infection.

Antigen Detection

Agglutination reaction, immunofluorescence and enzyme immunoassay are the most widely used immunoassays for antigen detection.

Agglutination Reaction

Agglutination reaction is applied to directly detect possibly existent antigen of a small quantity in blood, cerebrospinal fluid and other secreted fluid in human body at the early stage of bacterial infection. For instance, neisseria meningitidis can be directly detected in the cerebrospinal fluid collected from a patient with epidemic encephalitis. Agglutination reaction facilitates rapid diagnosis of infectious disease.

Immunofluorescence

Immunofluorescence is a microscopic examination which combines immunological specific reaction and fluorescent trace. It maintains high serological specificity and greatly improves the sensitivity of detection, thereby playing an important role in bacterial detection.

Enzyme-Linked Immunosorbent Assay (ELIZA)

In addition to detection of both pathogen and antibody, ELIZA can also be applied to identify bacterial metabolites. Almost all the soluble antigen-antibody reaction systems are detectable by ELIZA, with the detectable minimum value reaching ng or even lg. And both specificity and sensitivity are high.

In addition to the above immunoassays, countercurrent immunoelectrophoresis, immunoblot assay, and luminescence immunoassay can also be applied in clinical practice to detect pathogenic bacteria.

Antibody Detection

After human is infected by pathogenic bacteria, the immune system is stimulated to produce immune responses, and specific antibodies are then produced. The quantity of antibodies commonly increases along with the progression of infection, with manifestation of increased titer. Therefore, a known bacteria or its specific antigen can be used to detect the existence of the corresponding antibody in the serum as well as the dynamic changes of the titre. This facilitates the diagnosis of certain infectious diseases. And antibody detection is mainly applied in the diagnosis of infectious diseases caused by bacteria with strong antigenicity and a long illness course.

That the antibody titre is obviously higher than the normal level or the titre value at the convalescent stage is at least 4 times as high as that at the acute stage indicates a serological diagnosis with clinical significance.

6.2.4.2 Molecular Biological Detection

Nucleic Acid Hybridization

Hybridization occurs when two individual single-stranded DNAs bind complementarily to form a double-stranded DNA. Based on the process of hybridization, a specifically

sequenced DNA segment is prepared to serve as a labelled probe. Under certain conditions, it can hybridize with the denatured bacterial DNA in the specimen based on the base complementary pairing rule. Since the occurrence of hybridization can be detected via hybridization signals, the existence of the pathogenic bacterial genes in the specimen can be determined. Nucleic acid hybridization is a bacterial examination with high sensitivity, high specificity, simple operations and rapid result that can directly detect pathogenic bacteria in specimens. And the detection successfully avoids effects from non-pathogenic bacteria. Therefore, for those pathogenic bacteria that cannot be cultured or are difficult to be cultured, nucleic acid hybridization is of great significance for their detection.

Polymerase Chain Reaction (PCR)

PCR is applicable to detect pathogenic bacteria which cannot be precisely and immediately detected via conventional cultures, show low sensitivity to conventional cultures, or need a long culture time. For instances, the culture of mycobacterium tuberculosis consumes 2–3 months, which delays the diagnosis and immediate treatment. The infection of chlamydia trachomatis commonly shows no characteristic symptoms and the culture of pathogenic bacteria is challenging. Such pathogenic bacteria also include legionella, mycoplasma pneumoniae and rickettsia. And PCR is definitely a good choice for their detection. In addition, PCR is also widely used in bacteriotoxin detection. Since different bacteria produce different toxin, a specific primer can be designed and synthesized based on the specific toxin gene and the specific toxin gene segment can be amplified by PCR. Such a way shows high specificity and sensitivity. Currently, with the technological development of PCR, the fluorescent quantitative PCR overcomes the weakness of traditional PCR in relatively high rate of false positive and shows precise quantification.

Biochip

Biochip, as an emerging high-tech in the life science, has been rapidly developing in recent years. By establishing a mini biochemical analysis system on the surface of a solid chip via micromachining and microelectronics, biochip can process a large quantity of data precisely and rapidly to detect cell, protein, DNA and other biological components. The widely used biochips include gene chip and protein chip. The diagnostic biochip for pathogenic bacteria allows simultaneous detection of different pathogens in different specimens in one chip. Only with extremely small quantity of specimen, it offers a large quantity of diagnostic information within an extremely short period of time. Thereby, biochip technology provides a rapid, sensitive and high-flux way for clinical diagnosis of bacterial infection.

6.2.4.3   Bacteriotoxin Detection

Endotoxin Detection

Endotoxin detection is mainly applied for definitive diagnosis of Gram negative bacterial infection. Most of gram negative bacteria can produce endotoxin, which is released after death and lysis of the bacteria, with multiple biological effect. As an exogenous pyrogenic agent, the endotoxin can stimulate the white blood cells to release endogenous pyrogens, which then acts on the body temperature center to cause fever.

Limulus test is always applied for endotoxin detection, which shows high specificity to endotoxin generated by Gram negative bacteria but shows negative to non-endotoxin substances, Gram positive bacteria and virus toxin. The test also has high sensitivity, with (0.005–0.0005) μg/ml of endotoxin detectable. The operations are simple and the test result can be obtained within 2 h for typing of the pathogenic bacteria. The test result facilitates reasonable medication and early treatment.

Exotoxin Detection

Exotoxin detection can be used to identify an unknown bacteria and to distinguish toxigenic strain from non-toxigenic strain.

Internal Virulence Test

The toxic effect of bacterial exotoxin can be neutralized by the corresponding antitoxin. An animal fails to experience toxic symptoms after injection of extotoxin following antitoxin. Based on such a phenomenon, the production of corresponding exotoxin in the bacteria can be identified.

External Virulence Test

With strong antigenicity, exotoxin stimulates the organism to produce corresponding antibody. The serum with specific immunity to the bacterial exotoxin, selected as the antibody, should respond to the detected bacterial exotoxin (antigen) in vitro to verify the existence of the bacterial exotoxin.

In addition to the above examinations, the exotoxin produced by most bacteria can be detected by ELISA, such as staphylococcal enterotoxin as well as heat-labile toxin (LT) and heat-stable toxin (ST) produced by enterotoxigenic escherichia coli.

6.2.4.4   Animal Experiment

Animal experiment is an important part of the clinical bacteriological examination, and sometimes cannot be replaced by other examinations. It is mainly applied to isolate and identify the pathogenic microorganism, to determine the bacterial virulence, to prepare serum with specific immunity, to establish pathogenic animal model, to collect animal blood for bacteriological culture mediums, and to test the

safety, toxicity and therapeutic efficacy of biological products and some drugs. In addition, the bacterial virulence and immunogenicity may change via the passage in susceptible and non-susceptible animals. Animal experiment requires not only understanding about the classification of experimental animals, but also appropriate selections of experimental animal and inoculation way based on the purpose and demands of experiment. The widely used experimental animals include mice, guinea pigs, rabbits and sheep, while the commonly used inoculation ways include subcutaneous injection, intradermal injection, intramuscular injection, intraperitoneal injection, intravenous injection and intracerebral injection.

6.3 Immunodiagnosis

6.3.1 Immunodiagnosis of Parasitic Diseases

Immunodiagnosis refers to diagnosis by *in vitro* detection of antigen or antibody based on the immune responses in human body triggered by invasion of parasites, including intradermal test serological test. The intradermal test is selected for initial screening of patients due to its low specificity. The serological test is applied to detect specific antigen or antibody via different examinations, with specific antigen positive indicating present infection while specific antibody positive indicating past or present infection. Therefore, serological test can be applied for diagnosis or to assist the diagnosis.

6.3.2 Classification of Immunodiagnostic Examinations for Parasitic Diseases

Based on immediately occurring allergy, the intradermal test is performed by injection of skin test antigen into the inner layer of epidermis. Positive or negative result can be obtained after observation of the skin mound. It is commonly applied for the diagnosis of helminthiasis or allergy induced by certain mites.

Serological diagnostic examinations include precipitation reaction, agglutination reaction, complement fixation test, immunofluorescence antibody assay, immunoenzymatic assay, radioimmunoassay, and immunoblotting. The serological diagnosis has evolved from serum sedimentation test and agglutination test to immunolabelling technique with efficiency requiring a trace amount of specimen and enzyme linked immunoblotting at the molecular level. These immunoassays can be applied to detect circulating antibody or antigen in infected human body, and are expected to be applicable for staging of an infection, identifying active stage of a new infection and assessing the therapeutic effi-

cacy. The serological diagnosis is playing an increasingly important role in the clinical diagnosis that the etiological diagnosis fails to. Almost all the immunoassays are applicable for the diagnosis of parasitic diseases but not always effective. In China, several serological diagnostic examinations have been developed for the diagnosis of parasitic diseases that serve as assisting tools for the diagnosis and provide reference for medication. These examinations have been gradually promoted to clinical application, and herein we introduce several commonly used immunoassays in clinical practice.

6.3.3 Immunodiagnostic Examinations

6.3.3.1 Intradermal Test

Basic Principle
The skin test antigen is injected into the inner layer of epidermis. A positive or negative result can be obtained by assessing the skin mound. It is mainly applied to diagnose helminthiasis or allergy induced by certain mites.

Advantages and Disadvantages
The intradermal test is rapid and requires simple operations and a short period of time. Its positive detection rate generally reaches above 90 % but with low specificity due to cross reactions among different parasitic diseases. In some cases, the patients always showed positive after being treated for years. Therefore, the result of intradermal test fails to serve as the basis for definitive diagnosis and for assessing the therapeutic efficacy. It is only applicable to screen the suspected cases in affected region.

6.3.3.2 Precipitation Test
With appropriate quantity of electrolytes, soluble antigen (such as exotoxin, endotoxin, lysate of bacteria, soluble antigen of virus, serum, tissue exudates) binds with corresponding antibody to form whitish deposits that are observable by naked eyes. The procedure is known as precipitation test. For instance, circumoval precipitin test (COPT) is one of the most common and effective serological tests for the diagnosis of schistosomiasis. And the test is based on the mechanism of specific immune response in human body to the antigen, eggs of schistosoma. Mature miracidium in schistosoma eggs can secrete soluble egg antigen (SEA), which exudates from micropores in the eggshell to attach to the surface of eggshell and binds to the antibody in the to-be-detected serum. The antigen-antibody complex deposites are thus formed around the eggshell, which are shown as bubble like or finger shaped deposits on the surface of eggs under a microscope. Such a finding indicates positive. In the serum of a healthy human body, no specific deposits can be observed

around eggs due to absence of corresponding antibody, which is defined as negative. Based on the perioval precipitation rate (the number of eggs with precipitation in per 100 eggs, which is calculated by the number of positive eggs/the number of observed eggs × 100 %), the COPT of examined serum can be assessed as positive or negative. Based on the size of deposits, the intensity of COPT can be understood. Currently, the test has been improving with favorable diagnostic efficacy and is applicable for population field application. The processing of eggs includes formaldehyde processed frozen dry egg antigen and heat processed ultrasonic dry egg antigen.

6.3.3.3   Indirect Heamagglutination Assay

Basic Principle
Indirect heamagglutination assay (IHA) is one of the agglutination tests, which is based on the biological mechanism that antigen binds to corresponding antibody to form complex whose agglutination, if electrolytes exist, can be observed as small pieces of deposits. Based on the produced agglutination, the existence of antigen or antibody can be detected. The procedure is known as the agglutination assay. The agglutination assay based on direct binding of granular antigen to antibody is known as direct heamagglutination assay. As for the indirect heamagglutination assay, a soluble antigen or antibody is firstly adsorbed to the surface of an immunity independent carrier particle with certain size, followed by its reaction with corresponding antibody or antigen. Under appropriate conditions agglutination of the carrier particles occurs along with the specific binding of antigen with antibody, showing observable agglutination. The commonly used carrier particles include red blood cells (O shaped human red blood cells or sheep red blood cells), polystyrene latex particles, kaolin, ion exchange resin, and collodions. The test with red blood cells selected as the carrier particles is known as indirect heamagglutination test.

Categorization
IHA has been applied for diagnosis and epidemiological studies of many parasitic diseases. It can be categorized into the following 4 types, all of which are for detection of antigen or antibody with high sensibility and certain specificity.

Positive Indirect Heamagglutination Test
An antigen is firstly adsorbed to the surface of red blood cells, followed by detection of the unknown serum antibody with a known heamagglutination antigen. Such a test is known as positive indirect heamagglutination test.

Reverse Indirect Heamagglutination Test
A specific antibody is used to sensitize red blood cells for detection of the antigen in the specimen.

Indirect Heamagglutination Inhibition Test
In the test, antigen sensitized red blood cells are used to detect the corresponding antibody. Specifically, the corresponding antibody is firstly added into the specimen, followed by addition of sensitized red blood cells after a certain period of time. If antigen exists in the specimen, the antigen binds to the antibody, with no agglutination after addition of the sensitized red blood cells. If no antigen in the specimen, agglutination is thus observable.

Reverse Indirect Heamagglutination Inhibition Test
In the test, antigen sentisized red blood cells are used to detect the corresponding antibody in the specimen. The corresponding antigen is firstly added into the specimen, followed by addition of the sensitized red blood cells. If antibody exists in the specimen, the antibody binds to the antigen with no agglutination. But if no antibody, agglutination is observable.

Advantages and Disadvantages
IHA has been widely used in the diagnosis of parasitic diseases and epidemiological studies, with high sensitivity and certain specificity. For instance, the positive rate for the diagnosis of schistosomiasis japonica has reached up to 91.9–100 %, with a false positive rate of only 0.7–3.2 %. The frozen and dried sensitized red blood cells can be preserved for 1–2 years at a temperature of 4 °C. The necessary equipment is not so complicated and has been manufactured in China. The test procedures are simple and applicable in community based clinics and hospitals. However, the red blood cells prepared in different laboratories or the red blood cells of different batches may show variance in sensitivity and specificity. Therefore, the preparation of the red blood cells should be standardized and commercialized. In addition, certain non-specific response may occur, which is related to the quality of sensitized red blood cells and hemagglutination inhibition test can be performed to exclude the possibility of non-specific response if necessary.

6.3.3.4   Immunofluorescent Assay
The assay can be applied to detect the antibody in serum for the diagnosis of malaria, filariasis, toxoplasmosis, schistosomiasis, paragonimiasis, clonorchiosis, echinococcosis, and amoebiasis. It can also be selected to detect parasites in tissues such as leishmania, amoeba, plasmodium and T. gondii.

Basic Principle
Immunofluorescence method is an immunoassay, which is also known as fluorescent antibody labeling. An antibody or antigen is firstly labeled with fluorescein, followed by observation of the labeled fluorescence under a fluorescence microscope to analyze the trace of the antigen or antibody.

Fluorochrome

Certain substances catch and absorb light energy to be excited, but emit light energy in the form of electromagnetic radiation when they regain their baseline state, which is known as photoluminescence. If exposed to light with short wave length, such as ultraviolet light, these substances emit light with longer wave length (such as visible light) within an extremely short period of time, which is known as fluorescence. The substances capable of emitting fluorescence are known as fluorochrome or fluorescein that can be categorized into various types. But the fluorescein capable of labelling antibody should: (1) be capable of covalent binding with immunoglobulin to form stable conjugate; (2) have no impact on immunoactivity of the immunoglobulin and no obvious effect on the fluorescence efficiency after the covalent binding; (3) be simple, fast, safe and non-poisonous in labeling. Currently, the most commonly used fluoresceins include fluorescein isothiocyanate, tetraethyl rhodemin, and tetraethyl rhodamine isothiocyanate. Nowadays, immunofluorescent assay has been applied in the diagnosis of many parasitic diseases, such as schistosomiasis, trypanosomosis, trichinosis, toxoplasmosis, and leishmaniasis.

6.3.3.5   Immunoenzymatic Techniques

Immunoenzymatic techniques are recently developed enzymatic immunoassays with both high specificity and high sensitivity, and have been widely applied in immunodiagnosis of parasitic diseases, such as schistosomiasis, paragonimiasis, black fever, echinococcosis, toxoplasmosis, cryptosporidiosis, pneumocystosis carinii, cysticercosis, and amoebiasis. In addition to traditional ELISA, K-ELISA, ABC-ELISA, DOT-ELISA, and film ELISA have been developed for the diagnosis of such diseases as schistosomiasis, paragonimiasis, and clonorchiasis. These immunoenzymatic assays can be applied to detect antibody, circulating antigen, stool specimens, pus and antigen in other body fluids. Currently, the procedures for antigen preparation and the procedures in laboratory test need to be clarified and standardized.

Basic Principle

Immunoenzymatic assays are another type of immune labelling technique following immunofluorescence method. They are based on the principles of specific binding of antigen with antibody as well as highly efficient catalytic effect of enzyme on substrate with the enzyme as marker. After enzyme labelled antibody or antigen binds with corresponding antigen or antibody, complex of enzyme-labelled antibody and antigen is formed. When the enzyme meets the corresponding substrate, it catalyzes the substrate to decompose, followed by oxidization of the hydrogen donor to form colored substance. The emergence of colored substance objectively demonstrates the existence of enzyme. Based on the presence and concentration of colored substance, the existence of suspected antigen or antibody as well as its quantity can be speculated so as to diagnose qualitatively or quantitatively.

Categorization

Methodologically, immunoenzymatic assays can be categorized into two types. One type is applied to detect and localize the antigen or antibody in tissues and cells, which is known as immunohistochemistry or immunoenzymatic staining. The other type is applied to detect soluble antigen or antibody in various body fluids, which is known as enzyme linked immunosorbent assay (ELIZA). After preparation of specimen, immunoenzymatic staining is performed with the following procedures: inhibition of the endogenous enzymes and the following immunoenzymatic staining for examination. Its basic principle and procedures are the same as those of fluorescence antibody method, only with enzyme to replace fluorescein as the marker and emergence of colored substances in the substrate as positive. Routine immunoenzymatic staining can be further categorized in to direct and indirect staining. Direct staining is performed to label specific antibody with enzyme for direct detection of parasite or its antigen. After fixation of the specimen containing parasite or its antigen, the inside endogenous enzymes are firstly inhibited. After that, enzyme labelled antibody is directly added for its binding to the antigen in the specimen, followed by addition of substrate for coloration and microscopic examination. Indirect staining is performed by processing of the tissues or cells containing parasite or its antigen with specific antibody for binding of the antigen to the antibody. The unbinding components are cleansed and eliminated, followed by addition of enzyme labelled antibody for formation of antigen-antibody-enzyme-labeled-antibody complex. Finally, the substrate is added for coloration and microscopic examination. Although with additional one step, the indirect staining shows higher specificity and has gained wider application. The second enzyme labelled antibody can be replaced by staphylococcal protein A (SPA) or biotin-avidin system, which has also be successfully applied to detect many antigens and antibodys. Meanwhile, both specificity and sensitivity of the staining have been increased by varying degrees. ELIZA can be further categorized into solid-phase ELISA and homogeneous ELISA. Solid-phase ELISA is to chemically or physically link antigen or antibody to a solid phase carrier to prepare an immunosorbent for immunoenzymatic assay. ELIZA is the most widely used solid phase immunoenzymatic assay. Homogeneous ELISA is applied to directly detect antigen or antibody in the solution with no isolation of the free enzyme marker from the binding enzyme marker and no use of solid phase carrier. It is

mainly used to detect small molecular hapten such as hormone and antibiotics.

Enzyme and Substrate Used for Labelling

Horseradish peroxidase, glucose oxidase, acid phosphatase, alkaline phosphatase and β-galactosidase are commonly used enzymes for labeling, of which horseradish peroxidase has gained the widest application.

Enzyme Labeled Antibody

The activity and purity of enzyme play a crucial role in its labeling of antibody. The antibody is also required to be highly purified, more favorably by affinity chromatography. However, mice ascites can be directly labeled in labeling of monoclonal antibody. An ideal binding agent should be of high productivity, producing stable conjugate, showing no effect on activity of enzyme and antibody, producing no interfering substances, and simple operations. Currently, glutaraldehyde labeling and sodium periodate labeling are mainly adopted. The principle of glutaraldehyde labeling is the covalent binding of its aldehyde group to amidogen of immunoglobulin. Namely, one of the two active aldehyde groups of glutaraldehyde binds to amidogen of enzyme molecules, while the other binds to amidogen of immunoglobulin to form enzyme-glutaraldehyde-immunoglobulin complex. Sodium periodate labeling is mainly used to label HRP. Its advantages include high labeling rate and small quantity of unlabeled antibody. However, the conjugate with large molecular weight shows less favorable penetration through the cells than glutaraldehyde labeled antibody. Therefore, it should not be applied in immuno-electron microscopy.

6.3.3.6   Latex Agglutination Test (LAT)

With latex microparticle as the carrier, LAT is used to replace agglutination test using red blood cells. It is mainly applied to diagnose toxoplasmosis, cysticercosis, trichinosis, schistosomiasis, and echinococcosis, with favorable sensitivity and specificity. The procedures of LAT are simple and the test results can be rapidly obtained that is preferable for field application.

6.3.3.7   Immunoblotting Test (IBT)

IBT, also known as Western blotting, is a rapidly developed technique in recent years that integrates polyacrylamide gel electrophoresis, transfer electrophoresis and solid-phase ELISA. It has been applied for the diagnosis of schistosomiasis, teniasis, echinococcosis, paragonimiasis, pneumocystosis carinii, amoebiasis, and cysticercosis. With its ongoing rapid development, IBT is expected to be an effective diagnostic immunoassay with high sensitivity and specificity for the diagnosis of parasitic diseases and for identifying the stage of parasitic infections.

6.3.3.8   Immunochromatography (ICT)

ICT is a rapidly developed diagnostic technique in recent years, which is used to detect antibody or antigen. Antigen detection for plasmodium falciparum/plasmodium vivax, filaria bancrofti, and leishmania has gained clinical application. It is applicable for rapid clinical diagnosis and field studies due to its high sensitivity and specificity as well as its rapidity in obtaining results.

Generally speaking, with the rapid development of immunological technology, the immunodiagnosis of parasitic diseases has advantages of simple operations, micro quantity requirement of the specimen, rapidity, high accuracy and low cost. With the application of the high-tech, immunodiagnosis of parasitic diseases will be of greater clinical value.

6.4   Genetic Diagnosis

With the rapid development of modern life science and technology, the laboratory techniques in the diagnosis of parasitic diseases have been extended beyond etiological and immunological examinations into genetic diagnosis. Its application plays an important role in the laboratory diagnosis of parasitic diseases. The genetic diagnosis targets on the specific DNA fragment in genome of parasite, which shows higher specificity and sensitivity compared to etiological and immunological diagnosis. In this section, we briefly introduce the genetic diagnosis of parasitic diseases.

6.4.1   Nucleic Acid Probe

6.4.1.1   Introduction

Nucleic acid probe is a widely used technique in genetic diagnosis with high sensitivity and specificity that gains rapid development in recent years. It is based on the principle that two single-strand nucleic acids with certain homogeneity anneal to form double strands by pairing of complementary bases. Therefore, the under-detected sequence of nucleic acid can be detected with a known probe. Nucleic acid probe refers to a DNA or RNA fragment that is labeled by radionuclide or other markers and is thus capable of binding specifically with specific target molecule.

6.4.1.2   Procedure

Generally speaking, four operational procedures are performed, including selection of nucleic acid fragment as probe, labeling, hybridization, demonstration or detection of hybridization signal.

Types of Probe and Its Selection

Based on its origin and property, nucleic acid probe can be categorized into genomic DNA probe, cDNA probe, RNA

probe and artificially synthesized oligonucleotides probe. It also can be categorized into radiolabeled probe and non-radiolabeled probe based on its markers. The probe should be selected based on the following principles:

1. High specificity: For instance, exon is commonly selected as probe for human genome.
2. Simplicity in preparation: In simplicity of preparation, the single strand is superior to double strands with a more efficient hybridation.
3. Conveniency in detection and high sensitivity: When a short fragment is selected, it carries less marker with low sensitivity.
4. A general length of 17–50 bp: A longer fragment generally consumes longer period time for hybridization, while a shorter fragment shows lower specificity.
5. A probe containing 40–60 % G-C shows higher rate of specific binding.
6. Less than 4 repetitive bases: Intron is generally not selected as probe for eukaryocyte genome.
7. The homogeneity should be less than 70 % or less than 8 consecutive base in the non-target molecular area.

Labeling

An ideal probe marker should embed the following properties: (1) highly sensitive but low false positive rate; (2) no impact of the binding between marker and probe on specificity of base pairing; (3) no impact on the main properties of the probe, such as physicochemical properties, hybridization specificity and stability, as well as Tm value. In addition, the procedure should show high sensitivity and specificity, which requires simple procedures for labelling and detection and the labeled marker can be preserved for a long period of time. It also should not contaminate the environment and do no harm to human health, with a low cost. The applicable markers include radioactive markers such as ^{32}P, ^{3}H, ^{35}S and non-radioactive markers such as biotin, digoxin and enzymes. The radioactive markers are highly sensitive and specific, but their use requires special safety procedures in addition to their contamination to the environment and a short half-life period. Non-radioactive markers are free of contamination and special safety procedures, with favorable stability for prolonged preservation. However, they are inferior to radioactive isotopes in term of sensitivity and specificity. The labeling of probe can be categorized into in vivo labeling and in vitro labeling. In vitro labeling is more commonly applied, including chemical labeling and enzymatic labeling.

Hybridization

The molecular hybridization of nucleic acid is actually denaturation of double-stranded DNA and renaturation of two homogeneous single-strands. The molecular hybridization of nucleic acid can be categorized into solid-phase hybridiza-

tion and liquid-phase hybridization based on the medium and responsive environment. Solid-phase hybridization is achieved by first fixation of one nucleic acid chain on a solid support while the other chain is free in liquid. It has gained wide application due to the simplicity in operations. Liquid-phase hybridization, however, refers to the test procedure that two nucleic acid chains participating in hybridation are free in liquid, with rapid hybridation. Liquid-phase hybridation is commonly applied together with nucleic acid electron microscopy to study the homogeneity of different DNA and the relationship between mRNA and chromosomal DNA. Liquid-phase hybridization can be classified into Southern blotting, Northern blotting and Western bloting based on different target of identification. Southern blotting is employed to analyze recombinant DNA, recombinant plasmid and bacteriophage, while Northern blotting is used for qualitative and quantitative analysis of RNA. Western blotting is applied for qualitative and quantitative analysis of proteins and their interactive effect. According to the type of nucleic acid and approaches for detection, liquid-phase hybridization can also be categorized into Southern blotting, dot blot hybridization, in situ hybridization and Northern blotting.

Detection of Hybridization Signal

Approaches to detection of hybridization signal vary with the markers labeled on the probe. The probes labeled by radioactive isotope are shown on X-ray film via radiography, while the probes labeled by biotin are indicated by enzyme-chromogenic substrate via ABC system. The probe labeled by digoxin with enzyme labeling digoxin antibody is also indicated by chromogenic substrate.

6.4.1.3 Application of Nucleic Acid Probe in Diagnosis of Parasitic Diseases

In the diagnosis of parasitic diseases, probe is a specific nucleotide sequence of pathogen, which can be used to detect the existence of pathogenic parasite. And preparation of highly specific probe is the key procedure. Currently, parasite-based nucleic acid probes available include complete genomic DNA probe, kinetoplast DNA probe, recombinant plasmid DNA probe or bacteriophagic recombinant DNA probe, artificially synthesized oligonucleotide probe.

To date, concerning the detection of parasite, nucleic acid probe is mainly used to detect parasite in blood such as plasmodium, leishmania, trypanosome and toxoplasma gondii. Microscopy fails to detect these protozoa when the protozoal density in blood is low and, therefore, is not applicable in large-scale epidemiological studies. Although detection of antibody in serum can provide important information about protozoa infection, it fails to define the existence of viable protozoa due to detectable antibody long after the disappear-

ance of protozoa. With the development of molecular biology, the identification of pathogen via detection of nucleic acid emerges. Generally speaking, heterologous nucleic acid can be resolved rapidly in blood. Therefore, the finding of pathogenic nucleic acid in blood demonstrates the existence of viable pathogen.

6.4.2   Polymerase Chain Reaction (PCR)

6.4.2.1   Introduction

PCR is a technique for nucleic acid amplification in vitro which is developed in mid 1980s, with advantages of high sensitivity and specificity, being rapid, convenient and automatic as well as favorable repeatability. The target gene or certain DNA segment can be amplified into 100 thousands to one million times within several hours by PCR, which renders direct observation with naked eyes possible. PCR can amplify the target gene or certain DNA segment into enough quantity of DNA from a single hair, a drop of blood or even a single cell for analysis and identification. PCR technology is a revolutionary invention and milestone breakthrough in biomedical field.

6.4.2.2   Principle and Procedure

The fundamentals of PCR resemble to the natural replication of DNA, and its specificity depends on the oligonucleotide primers which complement with both terminals of target sequence. The procedures of PCR include denaturing, annealing and extending.

Template DNA Denaturation

After heated to the temperature of 93 °C for a certain period of time, the double-strands template DNA or double-stranded DNA formed by PCR amplification break down into single strand. The single strand then binds to the primer for another reaction.

Template DNA

The template DNA denatures into single strand after heating along with annealing of primer. When temperature reduces to about 55 °C, the primer binds to the complementary sequence of the single strand template DNA.

Extension of Primer

Under the effect of Taq DNA polymerase, the conjugate of template DNA with primer produces a new semiconservative replication strand by using dNTP as raw material and target sequence as template. The production is achieved via base pairing and semiconservative replication.

More semiconservative replication strands can be obtained when denaturing, annealing and extending, the 3 processes in PCR, are repeated. And these new strands act as template

for next cycle of replication. Each cycle of these 3 processes requires about 2–4 min and the target gene can be amplified to millions of times within 2–3 h. The quantity of cycles to reach the plateau level depends on template copy in the specimen.

6.4.2.3   Reaction Component

The basic system of PCR is composed of DNA template, oligonucleotide primer, dNTP, DNA polymerase and reaction buffer with necessary ions, all of which affect PCR reaction.

DNA Template

DNA template is also referred to as target sequence, which can be either single-stranded DNA or double-stranded DNA. The amplification efficiency of ring-closed DNA template is relatively lower than that of linear DNA. Therefore, it is rather better to choose linear plasmid as template. Protease, nuclease, DNA polymerase and proteins that can bind with DNA should not mix with template DNA. To some extent, PCR production increases markedly along with concentration of template DNA. However, steep concentration of template DNA can increase non-specific reaction. To guarantee specificity of the reaction, the concentration of genomic DNA template should be about 1 μg; and plasmid DNA template, about 10 ng.

Primer

Primer is the key material in specificity of PCR, which depends on the degree of complementarity between primer and template. Theoretically, based on any template DNA with known sequence, complementary oligonucleotide strand can be designed as primer. By PCR, template DNA can be amplified considerably in vitro.

Thermostable DNA Polymerase

In addition to classic Taq DNA polymerase, many thermostable DNA polymerases are found, such as Tth DNA polymerase, Vent DNA polymerase and Pfu DNA polymerase.

Taq DNA Polymerase

Natural Taq DNA polymerase is directly isolated from Thermus aquatic YT-1 strain, which has favorable thermal stability. Its half life period is 130 min, 40 min, and 6 min at the temperature of 92.5 °C, 95 °C, 97.5 °C, respectively, and the optimum temperature for catalyzing DNA synthesis is 72–80 °C. Taq DNA polymerase shows the following properties: directional polymerization of $3'-5'$, directional exonclease activity of $5'-3'$, reverse transcriptase activity, relatively weak non-template dependency, absence of directional $3'-5'$ exonclease activity.

In addition to natural Taq DNA polymerase, there is also modified Taq DNA polymerase. One is recombinational Taq

DNA polymerase which is acquired from the expression of colibacillus via genetic technology. The other is known as Stoffel segment that shows favorable thermal stability (Vainshtein et al. 1996). It was firstly obtained by Stoffel et al. via cutting off the N-terminal of natural Taq DNA polymerase.

Tth DNA Polymerase

Tth DNA polymerase is a thermostable DNA polymerase isolated from Thermus thermophilus HB8. It can effectively reverse transcribe RNA at a high temperature with the presence of $MnCl_2$. When Mg^{2+} is added in chelated Mn^{2+}, polymerization of Tth DNA polymerase increases which can enable synthesis and amplification of cDNA with one catalyzer.

Vent DNA Polymerase

Vent DNA polymerase is also known as Tli DNA polymerase, which is isolated from pyrococcus horikoshii at the vocalnic vent in deep sea. In addition to favorable thermal stability, it also has directional exonclease activity of $5'-3'$ and the function of correcting. Additionally, it can amplify template NDA that is larger than 12 kb.

Pfu DNA Polymerase

Pfu DNA polymerase is purified from pyrococusfurisus strain, with directional polymerization of $5'-3'$ and directional exonclease activity of $3'-5'$. Its fidelity in DNA catalysis is 12 times as high as that of Taq DNA polymerase and it also has favorable thermal stability. Its half life period at the temperature of 97.5 °C is more than 3 h.

The concentration of DNA polymerase is a key factor affecting PCR reaction, with an optimal concentration level for each PCR reaction. Excessive polymerase can reduce specificity of the reaction, while insufficient polymerase may affect the production of reaction. For instance, the optimal quantity of Taq DNA polymerase is between 0.2 and 2.5 units in the 50 μl PCR system.

Deoxy-Ribonucleoside Triphosphate (dNTP)

dNTP in PCR can be grouped into 4 types, including dATP, dGTP, dCTP and dTTP. The molar concentration of these 4 dNTPs should be equal, otherwise, the variance may induce misincorporation of polymerase to reduce the velocity of new strand synthesis. The concentration of dNTP depends on the length of the amplified segment, concentration of MgCl and concentration of primer. The final concentration is commonly ranges from 50 to 200 μmol/L. A high concentration of dNTP may produce error base incorporation, while a low concentration of dNTP may reduce the production of reaction. The concentration of dNTP should be adjusted to a pH value of 7.0 via addition of NaOH to guarantee a pH value of being not lower than 7.1 during reaction.

Clinically, dTTP is usually replaced by dUTP to prevent contamination from amplified product and control the false positive rate.

Buffer

The standard buffer in PCR is usually consisted of Tris–HCl, KCl, and MgCl with a Tris–HCl concentration of (10–50) nmol/L. Mg^{2+} is of great importance because its concentration may affect the activity of DNA polymerase and melting temperature of double-stranded DNA. As a result, Mg2+ has significant effect on the specificity and the production of PCR. Its lower concentration may reduce both the activity of DNA polymerase and the production of PCR, while its superfluous concentration may affect the specificity of PCR. The optimal concentration of Mg2+ is between 1.5 and 2.0 mmol/L. All of the DNA template and primer in PCR compound, phosphate group of dNTP as well as chelating agent such as EDTA can bind with Mg2+ to reduce the actual concentration of Mg2+.

6.4.2.4 Conditions for Reaction

Denaturation Temperature and Time

The denaturation of template DNA or PCR product in PCR is of great importance in its complete melting into single strands. Generally speaking, the higher temperature and longer time guarantee complete denaturation. However, too high temperature and prolonged time may affect the activity of Taq DNA polymerase. Therefore, denaturation temperature and time are commonly selected to be 95 °n for 30 s. The first circular denaturation of PCR may require more time due to the relatively longer template DNA.

Annealing Temperature and Time

Optimal denaturation temperature and time are the key points for the success of PCR, while annealing temperature and time affect the specificity of PCR. Lower temperature causes better renaturation, but with possible mismatch of primer and target DNA to increase non-specific binding, while higher temperature is disadvantageous for renaturation. Generally, the temperature for renaturation is about 55 °C.

Extension Temperature and Time

The extension temperature of primer is generally 72 °C due to the considerations about both the activity of Taq DNA polymerase and the binding of primer and targeted gene. Unfavorable extension temperature affects both the specificity and quantity of amplified product.

Cycle Number

The quantity of PCR amplified product is determined by the number of reaction cycles. The optimal cycle number is determined by the original concentration of target sequence

when the other parameters have been optimized. A low original concentration of target sequence requires more cycles of reaction. In the case of reduced enzyme activity or insufficient enzyme, more cycles are also necessary for effective quantity of amplification.

6.4.2.5   Detection and Analysis of Amplified Product

Gel Electrophoresis
After electrophoresis and ethidium bromide staining, the PCR product can be observed under a UV detector for preliminary judgement of its specificity. The size of PCR product segment should be in consistency with the predicted size.

Agarose Gel Electrophoresis
One to two percent of agarose gel is commonly employed for detection.

Polyacrylamide Gel Electrophoresis
Six to ten percent of polyacrylamide is better than agarose in gel electrophoresis, which has more concentrated strips and is applicable in scientific research and surveillance.

Enzyme Digestion Analysis
Theoratically consistent segment is obtained via corresponding enzyme digestion and gel electrophoresis based on restriction enzyme site. Enzyme digestion analysis can be applied to identify the PCR product, to type the target cells and to analyze their variability.

Molecular Hybridization
Molecular hybridization can provide sufficient evidence for the specificity of PCR product, and is an effective approach in detecting the base mutation of PCR product.

Southern Blotting
By using Southern blotting, a oligonucleotide strand is synthesized between two primers, which can be used as probe after being labelled to hybridize with PCR product. Southern blotting can be used to identify the specificity of PCR product, and can increase the sensitivity in detecting PCR product. In addition, it can also be used to understand its molecular weight and the shape of strip. Southern blotting is mainly used in scientific research.

Dot Blot Hybridization
The PCR amplified product is firstly dropped on nitrocellulose filter membrane or thin nylon membrane, followed by hybridization with inner oligonucleotide probe to observe the presence of stained spots. Dot blot hybridization is mainly used to identify the specificity of PCR product and to analyze its variance.

6.4.2.6   Application of PCR in the Diagnosis of Parasitic Diseases

During recent years, PCR has been applied to detect infection of plasmodium, amebic protozoa, tyoxoplasma gondii, trypanosome, leishmania, cryptozoite and giardia lambli. For some protozoal diseases, the quantity of pathogen is extremely rare and thus undetectable with conventional diagnostic examinations. PCR amplification is an approach to define the diagnosis, which provides findings that conventional etiological examinations fail to, especially in detecting parasite in tissues. For instance, in the diagnosis of trypanosomiasis disease, single polypide in blood specimen can be detected by PCR amplified and purified DNA probe. Currently, the diagnostic procedures of toxoplasmosis and falciparum malaria by PCR have been established in China. PCR is a promising and prospected molecular biological technology in the diagnosis of parasitic diseases.

6.4.3   Gene Chip Technology

6.4.3.1   Introduction
Gene chip, also known as DNA chip and DNA microarray, is a technique for the diagnosis of diseases based on analyzing the intensity and distribution of hybridization signals. A large quantity of DNA probe molecules should be firstly fixed on the support, followed by their hybridization with labelled sample. The intensity and distribution of hybridization signals are then detect for analysis. In recent years, gene chip technology has been continually improved and shows its application value in genetic diagnosis, gene expression research, genome research, discovery of new genes and detection of various pathogens.

6.4.3.2   Principle
Gene chip technology is a rapid, effective way in analyzing nucleotide sequence based on gene recombination technology. Firstly, a large quantity of DNA probe molecules are fixed on the support and subsequently hybridize with labelled sample. Via detecting the intensity and distribution of hybridization signals, gene analysis is conducted. On a gene chip of 1 cm^2 in size, necessary thousands of, even millions of genes can be fixed to form a gene array for synchronous detection of genes. In the gene chip technology, 4 major steps should be performed, including chip preparation, sample preparation, hybridization and detection of hybridization signal, and analysis.

6.4.3.3   Operational Procedures

Chip Preparation

Probe Synthesis
Probe synthesis is the first important step in gene chip preparation. The probes of gene chip include genomic

probe, cDNA probe and oligonucleotide probe. As for the genomic probe, a sequence which completely matches with the target sequence for detection or carries mutant site is firstly selected from genomic bank. After PCR amplification, a genomic probe is prepared. As for short oligonucleotide probe, it can be directly synthesized by DNA synthesizer. However, cDNA probe can be prepared by selection in cDNA bank with following PCR amplification based on the expressed mRNA of genes for detection. The selected sequence for PCR amplification should be close to 3′-terminal of cDNA, with a length of about 1 kb.

Fixation of Probe on the Surface of Carrier

Many approaches can be chosen to fix the probe on a solid support, which can be categorized into in situ synthesis and off-chip synthesis. In situ synthesis refers to synthesis of oligonucleotide probe on the surface of support, including in situ lithography synthesis, piezoelectric printing and molecular stamp in situ synthesis. By off-chip synthesis, pre-synthesized probe, cDNA or genomic DNA are directly placed on chip by a specific rapid off-chip robot, which is more commonly applied for large segment of DNA and sometimes oligonucleotide and even mRNA. The solid carrier used as gene chip mainly include solid flakes (such as glass, silicon chip, and ceramic chip) and thin films (such as nitrocellulose filter membrane, nylon membrane and polypropylene film). Glass slide or nylon membrane are usually employed as carrier of the gene chip.

Sample Preparation

The sample to be detected is mainly DNA/mRNA obtained from blood cells or tissues. Many sample molecules are needed in detection, and the sample is commonly amplified before labelling and analysis in order to improve the sensitivity of reading. As for the isolated genomic DNA, direct amplification by PCR can be performed, while as for mRNA, cDNA should be firstly prepared by using RT-PCR.

Many materials can be used to label the sample to be detected, such as Cy3, Cy5, biotin, and radioactive isotope ^{32}P. The labelling of sample refers to the process that dNTP carrying marker, such as Cy3 or Cy5-dNTP, biotin-dNTP, ^{32}P-dNTP, is interfused into target molecule sequence via amplification of DNA or cDNA. Otherwise, the dNTP carrying marker is interfused into the target molecule sequence during synthesis of primer, which is then used to amplify the target sequence, with the terminal of amplified product labelled. Fluorescence labeling is more commonly employed, which is based on the fact that the labeled molecules can be activated by laser to emit fluorescence within specific wave length. Therefore, the sample containing labeled molecules can be detected. This approach does not require labeling by isotope but with extremely high sensitivity and is applicable

for quantitative detection. Therefore, it is widely applied in labeling of samples on gene chip.

Hybridization

Hybridization refers to the reaction between fluorescence labeled sample with the probe on the chip to produce a series of information. Appropriate conditions can optimize reaction among biological molecules to reduce the mismatch rate. After hybridization, the matrix is inserted in a scanner to detect the mode of hybridization. The data of hybridization signal can be obtained when fluorescence signal labeled in the target is stimulated. The signal produced by matched probe and target is often stronger than mismatched probe and target. As the sequence and site of each probe in the matrix are known, the properties of target nucleotide can be identified by using the probe matrix.

Detection and Analysis of Hybridization Signal

Many approaches can be used to detect hybridization signal, such as fluorescence microscopy, mass spectrometry, chemiluminescence and optical fiber, among which fluorescence microscopy has gained the widest application.

As for detection technique based on nucleic acid hybridization, the main process of fluorescence detection is described as follows: The target sequence or sample that has been amplified is firstly labeled by fluorescence, followed by hybridization with the large quantity of probes on the chip. After the non-hybridized molecules are flushed away (the procedure can be omitted when real-time fluorescence is used), a fluorescence microscope is used to scan the gene chip to collect fluorescence intensity of each site for comparative analysis. As double strands with normal base pairing show more favorable thermal stability than double-strands with mismatched base pairing, fluorescence signal intensity of the double strands with well matched base pairing is 5–35 times as high as that double strands probe with one or two mismatched base pairing. Additionally, the intensity of fluorescence signal shows certain linear relationship with the quantity of target molecule in the sample. Therefore, precise detection of fluorescence signal intensity can demonstrate the specificity and intensity of hybridization. The approaches for quantitative analysis of fluorescence intensity from each site of highly dense probe matrix mainly include confocal laser scanning microscopy and charge-coupled camera.

6.4.3.4 Application of Gene Chip in the Diagnosis of Parasitic Diseases

Due to its simple operations and simultaneous sensitive rapid detection of thousands of genes, in addition to its highly specific stable automatic information, DNA gene chip technology is believed to be a revolutionary milestone in genetic information analysis. Furthermore, it provides a prospected future for academic research and clinical application in

parasitology. Its present or potential application fields include categorization and evolution of parasites at the genetic level, relationship between parasites and their environment, research in vaccines for protection against parasites, molecular diagnosis of parasitic diseases, research in drug resistance of parasites and new drug development.

6.4.4 DNA Sequencing

6.4.4.1 Introduction

DNA sequencing is to map out the sequence of 4 chemical bases that comprise a stand of DNA, namely first grade detection of nucleic acid sequence, which is an important technology in modern molecular biology. The basic procedures in routine sequencing include: (1) to process the DNA molecules to be detected to obtain a series of one nucleotide curtate DNA molecular complex; (2) to isolated these DNA molecules by gel electrophoresis to form a stripe in cascade shape arrangement for readings of each DNA base.

6.4.4.2 Principle

Approaches to DNA sequencing include the dideoxy chain termination proposed in 1977 by Sanger et al. (1981) and chemical degradation proposed in 1977 by Maxam and Gilbert. The principle of Sanger DNA sequencing is to use a DNA polymerase to extend primer that binds to the template with sequence to be detected until termination of nucleotide by interfusing one strand. Each sequencing is consisted of 4 independent reactions in a series. And each reaction contains all 4 dNTPs with addition of limited quantity of different ddNTP. Due to the absence of necessary 3-OH group in ddNTP extension, the extending oligonucleotides are terminated selectively at site G, A, T or C, which is determined by corresponding dideoxy in reaction. The relative concentration of each dNTP and ddNTP can be adjusted to obtain a chain termination product with bases ranging from hundreds to thousands. They share common starting point, but terminate at different nucleotides, and can be separated into fragments in different sizes via high resolution denaturing gel electrophoresis. After that, X-ray film autoradiography or non-isotope labeling can be applied for their detection. The chemical modification sequencing proposed by Maxam and Gilbert is based on the fact that certain chemical reagent can induce base modification at specific site (or specific type of base) in DNA strand, with following base shedding or replacement and finally occurrence of specific rupture. The rupture of different molecules at different sites can produce a series of DNA fragments in different sizes, which are then separated by gel electrophoresis. Before analysis, 5′-terminal of DNA is labeled by isotope for readings of DNA nucleotide sequence after autoradiography.

6.4.4.3 Next-Generation and Third-Generation of Sequencing Technology

With the development of science and technology, traditional Sanger sequencing is not sufficient for academic research. Both genome resequencing of model organism and genome sequencing of non-model organism require a sequencing technology with lower cost, higher flux and rapid sequencing. As a result, the next-generation sequencing emerged. The core of next-generation sequencing is sequencing by synthesis, namely capturing newly synthesized terminal labeling to determine DNA sequence. Currently, the technological platforms available include Roche/454 FLX, IIIumina/SolexaGenome Analyzer and Applied BiosystemSOLID system.

Researchers from University of Washington and other institutions have made a breakthrough in new-generation sequencing using nano-biotechnology. This new approach can provide individualized gene sequencing blueprint for more efficient individualized medical care for patients with cancer, diabetes or certain substance abuse. This achievement has been published on PNAS, which is known as the next-next-generation sequencing or the third-generation sequencing. The new approach is based on singular molecule reading technology via nanopore, which is different from the next-generation sequencing and can read information directly, rapidly and simply. The next-generation sequencing requires the aid of fluorescence or chemiluminescent material to read DNA polymerase or DNA ligase for indirect sequencing via detecting optical signal released during linkage of base into DNA strand.

6.4.4.4 Application of DNA Sequencing in the Diagnosis of Parasitic Diseases

In recent years, with maturation of DNA sequencing and improvement of automatic sequencer, the sequencing technology has been greatly improved in speed and accuracy. Different gene code sequences in the living organisms are the basis to distinguish their different species. Studies on DNA sequencing of paragonimus and other parasites have been conducted in China and DNA sequencing will be widely employed in the detection of parasites in the future.

6.4.5 Ribosome Display Technology

6.4.5.1 Introduction

Ribosome display technology (RDT) is a new technology using functional protein for mutal screening that was improved in Plückthun laboratory based on polyribosome display technology. Via the procedures, the correctly folding protein and its mRNA can simultaneously bind on ribosome to form mRNA-ribosome-protein tripolymer. Therefore, the genotype and phenotype of target protein are integrated for

selection of antibody and protein bank as well as in vitro modification of protein. It has been successfully applied in screening some proteins with high affinity and specificity in binding to target molecule, such as antibody, polypeptide and enzyme, which is an important tool in screening of protein.

6.4.5.2 Principle

By amplification of DNA bank by PCR and simultaneous introduction of T7 initiator, binding site of ribosome and stem-ring structure, RDT is applied to transcribe DNA into mRNA, which is then translated in the cell-free translation system to display the translated product of target gene on the surface of ribosome. The formed mRNA-ribosome-protein compound composes the protein bank for ribosome display. After that, the corresponding antigens are chosen to screen the translated compound, with edetic acid to dissociate binded ribosome compound or with specific antigen to elute the whole compound, and to dissociate mRNA from the compound. Via RT-PCR, the next-round template for display is provided and the obtained DNA enters into the next round of accumulation. Some DNAs can be sequenced for analysis via cloning.

6.4.5.3 Development History of RDT

Kawasaki proposed to use similar approach to screen peptide ligand from peptide library. Previously early-stage peptide antibody had been used for immunoprecipitation and mRNA conjugating with ribosome and peptide had been successfully isolated. Mattheakis et al. (1994) put these forerunning ideas into practice for the first time and established polyribosome display technology for screening peptide ligands. By using this technology, they successfully screened peptide ligands of immobilized monoclonal antibody with an affinity constant of up to 10^9 (at the level of nmol) from a peptide library with storage capacity of 10^{12}. Gersuk et al. (1997) also successfully applied this technology to screen peptide ligands labeled by prostate neoplasm marker. During in vitro translation, the folding and translation of protein or peptide work synchronously. The natural peptide binding with ribosome also has enzymatic activity. These studies have demonstrated that folding of certain protein is not affected by ribosome-channel protein. Therefore, isolation of ribosome is not indispensable for protein to obtain native conformation. Based on studies mentioned above, Mattheakis's polyribosome display technology was greatly improved in Plückthun laboratory in 1997 and a new technology to screen complete functional protein, ribosome display technology, was established.

6.4.5.4 Operational Procedure

Transformation of Gene Fragment

In order to guarantee the effective transcription and translation of gene fragments, 5′ terminal should connect with T7 initiator sequence and SD sequence and the termination codon of 3′ terminal should be removed. Thus the ribosome can successfully remain on 3′ terminal of mRNA to connect protein and mRNA together. PCR extension is commonly performed for several times during transformation of gene fragment.

1. The necessary gene fragments and intervening sequences are amplified respectively.
2. Primer 4 and Primer 5 are used for connecting PCR to connect target gene and introns. Primer 5 contains SD sequence and Primer 4 contains 3′ terminal stem-loop structure sequence.
3. The PCR product undergoes PCR extension again, with Primer 6 containing T7 initiator sequence and 5′ terminal stem-loop structure sequence as well as the same downstream product. The 5′ terminal is connected with T7 initiator, stem-loop structure and SD sequence, while 3′ terminal integrates with intervening sequences and contains 3′ terminal stem-loop structure.

In Vitro Transcription and Translation

1. In vitro expression can be achieved with pronucleus originated E. coli S30 cell-free protein synthesis system, eukaryon-based downbreak fluid of rabbit reticulocyte and cell-free protein synthesis system for wheat embryo extraction. However, disputes exist concerning the question that which one is the most appropriate. In vitro transcription and translation can be conducted conjugately or respectively. Currently, in vitro protein translation system based on DNA template and in vitro coupled transcription and translation system based on RNA template are commercially available.

Transcription and translation should be conducted respectively in ScFv antibodies and other proteins containing disulfide bridges, because protein containing disulfide bridges can be only correctly folded under oxidizing condition. However, during transcription, T7 RNA polymerase requires β-mercaptoethanol with reducibility to maintain its stability. If target protein can be correctly folded under reducing condition, in vitro coupled transcription and translation system is recommended.

2. It is necessary to control the effect of RNase when in vitro transcription and translation is conducted respectively. VRC, transition state analog as inhibitor of RNase, can effectively inhibit nuclease and improve the display rate of E. coil ribosome. In addition, stem-loop structure of 3′ terminal and 5′ terminal can help mRNA to avoid the effect of exonuclease RNase II and endonuclease RNase E.

Affinity Screening

The mixture produced by translation is diluted by 4 times to terminate the reaction after the completion of in vitro translation. The concentration of Mg^{2+} in reagent remains consistently at 5 mmol/L to stabilize mRNA-ribosome-protein ternary complex for either direct application in screening experiment or storage at the temperature of 4 °C. The ribosome complex can be stabilized for at least 10 days at the temperature of 4 °C, but with gradually decreased productivity. During in vitro screening, Nonspecific reaction between antigen and antibody can be reduced when 1–2 % skim milk powder and 0.2 % heparin are added during in vitro screening. In addition, heparin can also inhibit nuclease.

Affinity Screening

The affinity screening can be categorized into solid-phase screening and liquid-phase screening, specifically ELISA and paramagnetic particle method. Hanes et al. (2001) believed that the antigen coats the plastic surface in ELISA, whose hydrophobic effect may affect spatial conformation of attachment protein and the screened antibody molecules are unable to identify the natural epitope of antigen. They also argued that paramagnetic particle method is to connect the catching label such as biotin with the antigen, with capturing of the label by using streptavidin beads after the formation of antigen-antibody complex for affinity screening.

Separation of mRNA

After screening, icy buffer containing 20 mmol LEDTA is added to elute mRNA, and the eluted mRNA is then processed by Dnase I to remove the residual DNA template. T7 initior is introduced again via RT-PCR and the necessary components for ribosome display such as SD sequence are used in next-round display. Otherwise, the components are directly used in Northern hybridization to assess the screening efficiency. The target genes acquired from the final-round screening are connected with plasmid, followed by transfer to E. Coli to obtain singular target clone. The single-strand antibody molecules are further expressed in vitro or in vivo (secreting type or inclusion body type) to identify their activity.

Oriented Molecular Evolution in Vitro

When ribosome display technology is used to screen non-variant library, mutation and recombination technology can be introduced via error PCR or DNA shuffering to increase the molecular diversity. Therefore, the possibility in obtaining target molecules with favorable affinity and stability as well as being capable of increasing enzyme activity can be greatly improved. Plückthumx's team has successfully screened 1.1 nmol ScFV with high affinity by using ribosome display technology. Subsequently, they increased its affinity by introducing DNA rearrange technique.

6.4.5.5   Application of RDT in the Diagnosis of Parasitic Diseases

RDT is performed completely in vitro, with simplicity in library establishment, large storage capacity, more molecular diversity, simplicity in screening, and no pressure in selection, compared with phage display technology or saccharomycetes display technology. In addition, the affinity of target protein can be improved by introducing mutation and recombination technology. Therefore, we believe it is a powerful approach in large-scale library screening and acquisition of molecular evolution and prospect its application in the diagnosis of parasitic diseases.

6.5   Pathological Diagnosis

The etiological examination of parasitic diseases is performed to morphologically identify the infected parasite in different stages of its development for clinical diagnosis. Different parasites gain their access into human body via diverse routes, reside at different parts within human body, and affect different organs and tissues. Therefore, all the procedures including collection of suspected specimen, identification of polypide and understandings about the possible parasitized site are of great importance for the etiological examination of parasitic diseases.

6.5.1   Site with Possibly Detectable Parasite

Generally speaking, each species of parasite has relatively fixed and specific parasitizing site, for instances, plasmodium parasitizes successively in hepatocytes and erythrocytes; and Paragonimus westermani parasitizes in lungs. However, many parasites may also be present at sites other than the common parasitizing body parts to cause ectopic parasitism. For instances, in addition to the mesenteric vein, schistosome may also grow in lungs, brain and other body parts to cause ectopic damages; protozoa parasitizing in blood or blood cells may spread to any part of human body along with blood flow to cause multiple organ damages or systematic extensive lesions, such protozoa as plasmodium, Leishmania donovani and Trypanosoma; parasites residing in the intestinal tract generally are confined to the digestive system to cause gastrointestinal symptoms, but the larvae of a notable proportion of intestinal nematodes may migrate inside the body via the blood flow, such as roundworm, hookworm and strongyloides stercoralis. The parasites residing in tissue may parasitize in different organs and tissues and incur inflammation and space occupying symptoms at the corresponding sites.

6.5.2 Selection of Methods and Sites for Specimen Collection as Well as Ways for Parasite Detection

Standardized procedures for specimen collection and processing are crucial to the accuracy in identifying parasitic infection. One organ may be infected by different species of parasites, characterized by variance in clinically manifestations. Therefore, the pathogen should be detected to obtain definite diagnosis for effective treatment of parasitic disease. The successful detection of parasite is based on knowledge about possible parasite at different sites, appropriate approaches in specimen collecting, and appropriate processing of the specimen.

The specimens collected for detection of parasite are diverse, ranging from excretions, such as stool, intestinal drainage and urine, and secretions, such as sputum, cerebrospinal fluid, bile fluid, tears, prostatic fluid and vaginal discharges; to tissues from different organs and different body parts for biopsy, such as specimens collected during surgery, histocytes harvested from puncture, skin scrapings and pus from necrotic and liquefied tissues. In addition, approaches for collecting different specimens via different routes may present varying difficulties. For instances, the collection of stool and urine specimens as well as their volume control are comparatively easy, while by needle aspiration of tissues for biopsy, only limited quantity of tissues can be obtained and the patients commonly experience pain. In addition to skillful practice of the procedures for specimen collection, timely processing of the specimen is also of great importance. The factors, such as selection of fresh or fixed specimen, selection of fixation fluid, approach for staining and the staining agent, selection of refrigerated or frozen specimen, procedures in specimen processing and the duration of preservation, all show effect on the result of etiological detection in different degrees. Some of these factors even directly affect the success of polypide detection or correct identification of the parasite if detected.

An appropriate selection of examination methods guarantees accurate diagnosis of parasitic disease. The parasite examinations are generally categorized into etiological examination, immunoassay and molecular biological examination, all of them have diverse specific methods and are of different values in identifying different species of parasite. For helminthic infections, successful detection of eggs, larva and/or imago in various clinical specimens, especially intestinal specimens, can define the diagnosis. For instances, by direct stool smearing or microscopic observation, the finding of roundworm, whipworm and hookworm eggs can define the diagnosis. As for the protozoa, because their polypides are minute ranging from only 1.5 μm (microsporidia) to 80 μm (balantidium coli), and some even grow within cells, the definitive diagnosis requires smearing and staining, or even special staining. For some parasites, the species can be defined by biochemical and molecular biological techniques. For instances, plasmodium infection can be defined by microscopic examination after blood collection, blood smearing and staining. As for the microsporidia, a definitive diagnosis requires electronic microscopy or molecular biological examination such as PCR. Different approaches in examining the same specimen show different sensitivity and specificity to the polypide. Therefore, the selection of appropriate examination is paramount to successful detection of polypide and accurate identification of parasite from the suspected specimen.

References

Gersuk GM, Corey MJ, Corey E, et al. High-affinity peptide ligands to prostate-specific antigen identified by polysome selection. Biochem Biophys Res Commun. 1997;232(2):578–82.

Hanes SD, Herring VL. Gentamicin enzyme-linked immunosorbent assay for microdialysis samples. Ther Drug Monit. 2001;23(6):689–93.

Mattheakis LC, Bhatt RR, Dower WJ. An in vitro polysome display system for identifying ligands from very large peptide libraries. Proc Natl Acad Sci USA. 1994;91(19):9022–26.

Sanger BKF. Pioneer of primary sequences. Biosci Rep. 1981;1(1):1–2.

Vainshtein I, Atrazhev A, Eom SH, et al. Peptide rescue of an N-terminal truncation of the Stoffel fragment of taq DNA polymerase. Protein Sci. 1996;5(9):1785–92.