The Pathogen Isolation of Mycobacterium Tuberculosis and Identification of Infectious Disease Mechanism in Mice

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Abstract
The threat of tuberculosis to human health is becoming more and more serious, and its characteristics of strong infectious and difficult detection in the early stage make it impossible to guard against. The research on the pathogen isolation of mycobacterium tuberculosis and identification of infectious disease mechanism in mice help people to further understand the relationship between mycobacterium tuberculosis and biological organism, and diagnose tuberculosis earlier.

The method of sputum separation culture was used in this paper, with convenient and low cost, and reliable results can be obtained. The positive rate of bacterial growth could be improved by the re culture of the growing colonies, and the colony growth could be seen in 2-4 weeks, and the results were confirmed by microscopic examination.

Through the determination of PDD in mice injected with mycobacterium H37Rv, the nylon wool column separation method was selected, which improved the purity of T cells, the ratio of living cells and the recovery rate. Through experiments, the recovery rate of T cells was 30%, and the ratio of living cells was 93%. The results show that the ratio of living cells is high. In the experiment of the proliferation and transformation of T lymphocytes in mice, it can be seen that the stimulation value of the immune group is higher than that of the normal group, which shows that the cell immunity of anti-tuberculosis has occurred in mice with the maximum stimulation value of 3.04.

Keywords: Tuberculosis, Mycobacterium tuberculosis, isolation and identification, infectious diseases.

Introduction

During the period from 2011 to 2015, 4 million 270 thousand patients with pulmonary tuberculosis were found in China, hundreds of thousands of patients die each year because of tuberculosis, and tuberculosis is a serious threat to the health of our people[1]. Because TB is difficult to be diagnosed at the initial stage, patients often fail to timely treatment. When most of the patients are diagnosed later, the disease has entered the middle and late stage, with the lack of effective cure means for treatment[2].

The study on the pathogen separation of mycobacterium tuberculosis can explore the effective and rapid diagnosis method, improve the diagnosis rate and reduce the risk of tuberculosis to human health. At the same time, the analysis of the infection of mycobacterium tuberculosis in mice is helpful for the further study of the infection of mycobacterium tuberculosis and the development of the survival and development in the living body, which is very important for human to fight tuberculosis.

Tuberculosis and Mycobacterium tuberculosis

Tuberculosis: Tuberculosis is a mycobacterial infection caused due to a chronic infectious disease of human and animal diseases, its mode of transmission is mainly depending on the patients with respiratory tract sputum cases of tuberculosis spread. To the patients with tuberculosis infection, there will be a variety of tissue and organs such as nuclear nodules and cheese necrosis and other pathological changes[3]. According to WHO statistics, 9 million 600 thousand cases of tuberculosis patients had been found newly in 2015 all over the world's, the incidence rate reached 133/10 million, and global tuberculosis infection was about 2 billion people[4].

Tuberculosis is a huge threat to human health, and it has seriously restricted the development of animal husbandry[5]. China is a country with high incidence of tuberculosis, and there are hundreds of thousands of people die because of tuberculosis each year. Taking the number of tuberculosis patients in our country during 2006 -2010 for example (see Table 1), in 2010 the number of people diagnosed was as high as 3 million 267 thousand, 242.7 people per million people with tuberculosis[6].

The main reasons for the prevalence of tuberculosis are the flow of people, not timely scientific treatment of sick animals and the mixed infection of HIV. To carry out the prevention and treatment of tuberculosis, we need to increase financial input and scientific research, reduce the threat of tuberculosis to human health[7].

As early as 1882, scientists Koch Robert discovered the bacillus. The pathogens of tuberculosis are the three species in the genus mycobacterium, that is, mycobacterium tuberculosis, mycobacterium tuberculosis and mycobacterium tuberculosis, and mycobacterium belongs to prokaryotes[8]. When the tuberculosis bacteria invade the

organism, it will be swallowed by macrophages, and be brought into the part of the lymphatic tissue, so that bacteria stay here to form a primary lesion, resulting in tuberculosis[9]. This kind of granulation is further developed into tumor-like nodules and it will spread to the cavity of the capsule, thereby forming the necrotic foci, which is difficult to be cured. If the resistance of infected body with mycobacterium tuberculosis is weak, bacteria will spread along the lymphatics, become secondary lesions[10].

Table 1
Statistics on the number of Chinese patients with pulmonary tuberculosis in 2006-2010

Particular Year	2006	2007	2008	2009	2010
Number of cases (10 thousand)	289.3	320.4	326.1	329.4	326.7
Patient rate (/100 million)	223.4	244.8	246.9	248.3	242.7

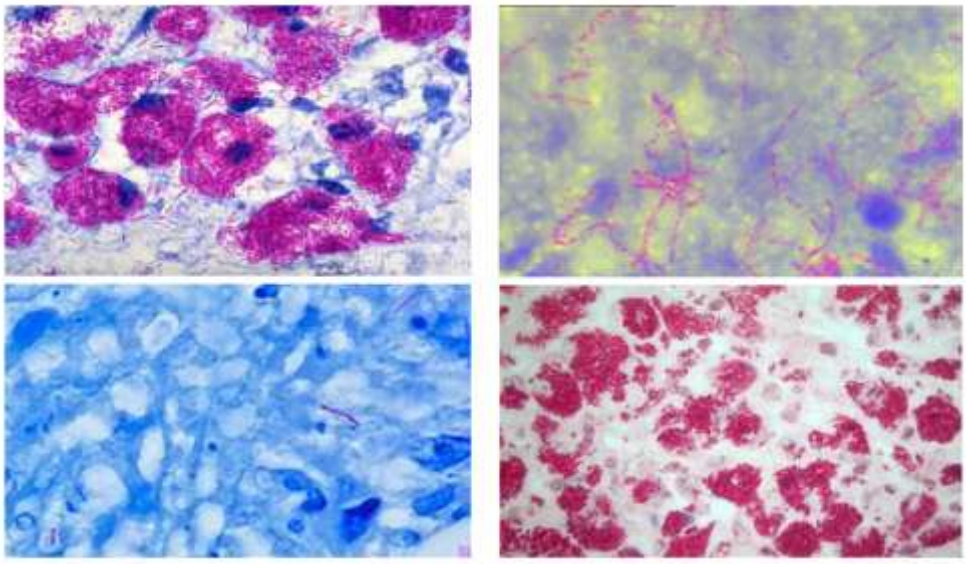

Fig. 1: Mycobacterium tuberculosis

When the bacteria enter into the bloodstream, they spread and develop into a systemic form of tuberculosis that forms the focus of other tissues and organs[11]. At this time, the body's lungs and lymph nodes will diffuse deposition, continue to develop, and ultimately form purulent calcification or cheese like necrosis. The initial immune response of tuberculosis is T cells, and the number of bacterial invasion directly shows the severity of the disease. Its main route of transmission is the spread of droplets, but eating water and food contaminated by bacteria can also cause lesions in the digestive tract[12].

When people infected with tuberculosis, the illness is difficult to be found in early stage, but the symptoms of powerless, loss of appetite, insomnia, fever, and night sweats will appear. When the illness becomes serious, the patient's temperature will rise, there will be blood in the sputum expectoration. Because of the small blood vessel injury, there will be hemoptysis. Severe hemoptysis may result in hemorrhagic shock or asphyxia caused by vascular occlusion[13].

When pleural thickening or fibrosis appears in the lung lesions, symptoms of subsidence, tracheal displacement and emphysema will appear in the chest, and invasive pulmonary tuberculosis can also be found chest radiography[14]. In the clinical, the more common is tuberculosis, lymph tuberculosis, intestinal tuberculosis and tuberculosis, and sometimes there will be genital tuberculosis, tuberculosis, etc.[15]

Mycobacterium tuberculosis: Mycobacterium tuberculosis of different forms is also different in morphology. Mycobacterium tuberculosis is straight or slightly curved, elongated or parallel to a single or parallel arrangement, and there are branching, dominated by rod. The morphology of mycobacterium is more, and the cell is small and short. The strain of mycobacterium tuberculosis is short and thick, and the staining is not uniform[16]. The average width of the bacteria is 0.2-0.5 μm, and the average length is 5-15 μm, both ends of blunt round, no bacteria and spores, no movement, positive Gram stain, which is shown in fig.1.

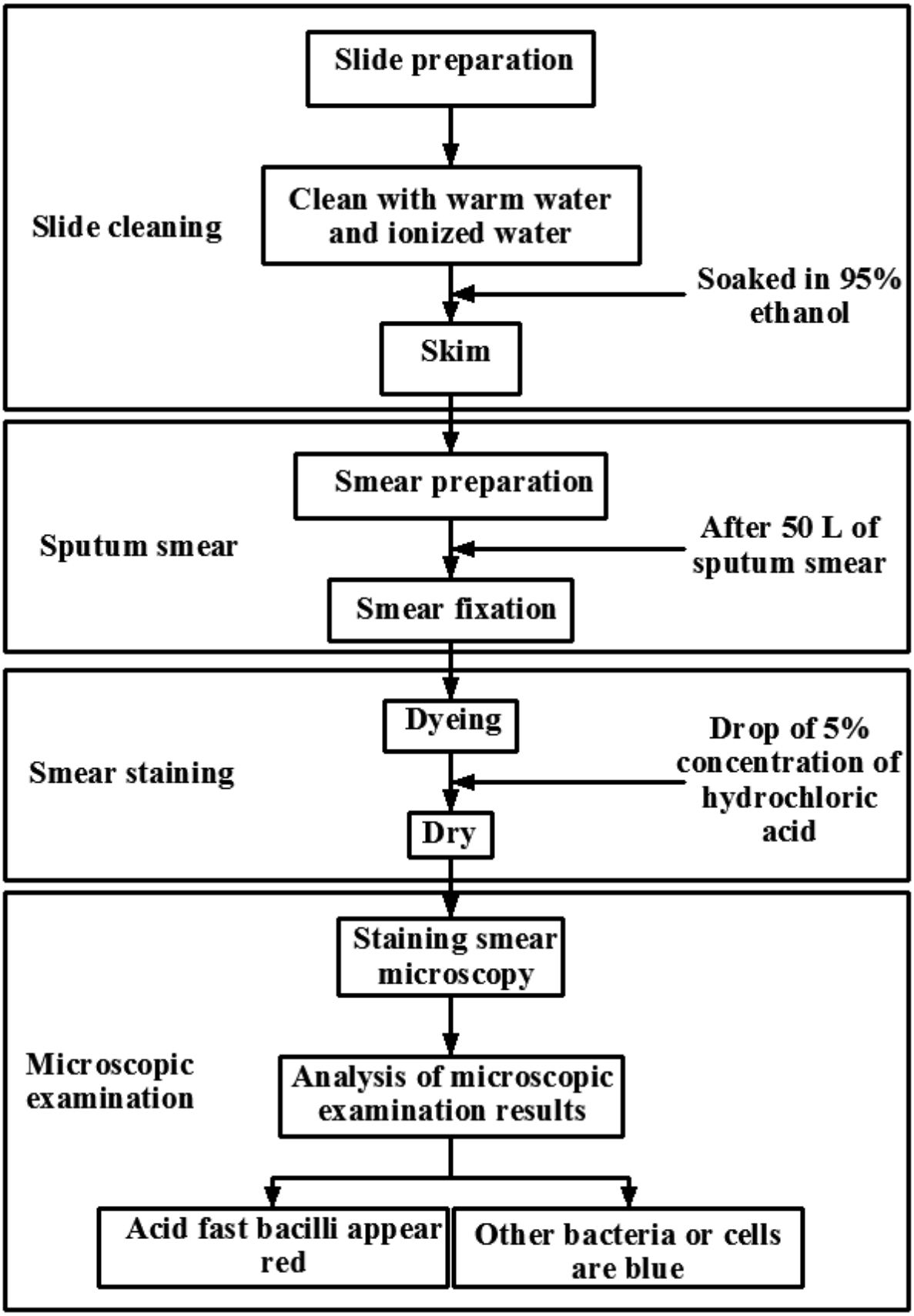

Fig. 2: Sputum smear microscopy and flow chart

The cell wall of the bacteria is not only a peptide, but also a special. Because of this, the general method of dyeing is difficult to color. Because of its characteristics of acid fast stain, it is also known as the acid resistance of mycobacterium[17]. There is a layer of capsule in the outer layer of mycobacterial cell wall, thick and difficult to be found, and its main role is to protect the mycobacterium tuberculosis[18]. Mycobacterium tuberculosis will have an effect on the synthesis of the peptide in the cell wall after the induction of serine and penicillin, and isoniazid will influence on the synthesis of branched acid, thus becoming negative acid fast stain.

Mycobacterium is a kind of special aerobic bacteria, and its growth temperature is about 37 degrees Celsius. When it is below 30 or above 42, it will inhibit the growth or non-growth of the bacteria. Due to the high lipid content of the Mycobacterium, it has an impact on the absorption of nutrients and the growth of the bacteria is slow[19]. In the nutrient rich medium, the bacteria split generation time is about 5h, and in general, it needs 18-24h. Commonly used medium has the improved solid culture medium, in which malachite green can inhibit its growth and facilitate the separation and cultivation, and egg yolk and glycerin can stimulate the growth of bacteria. The growing strain presents the particle, cauliflower or nodular shape, rough surface, irregular edge, yellow or milky white[20].

Due to the lipid in the cell wall of the mycobacterium, the bacteria have a strong ability to adapt to the environment, but they are very sensitive to the ethanol, and the bacteria will die within 2min in the concentration of 70% ethanol. In the sputum, bacteria can exist for 2-8 months, because the sputum can enhance the resistance of Mycobacterium tuberculosis. Bacteria can survive in the water for 5 months. When the temperature exceeds 63 degrees Celsius, the bacteria will die within 15min, boiling can kill the bacteria, and therefore the disinfection can be carried out by high temperature.

Experimental materials and methods

Pathogen isolation of mycobacterium tuberculosis: The sample used in the separation experiment is the sputum of the patients with suspected tuberculosis in a hospital from December 2014 to September 2015. Human positive and negative serum and the purified RV0899 protein are provided by Ruijie Company in Qingdao. The serum to be detected is provided by the other three hospitals. And the strain is stored in the laboratory of WH disease prevention and control center.

The required test agents are from a chemical company in Beijing, including analytical reagent grade or domestic reagent of carbolic acid, glycerin, sodium chloride, Lushi methylene blue, potassium dihydrogen phosphate, sodium hydroxide, sodium acetate, hydrochloric acid and complex red. Culture tube, centrifuge tube, inoculation ring, sealing film, grinding rod and other consumable materials are also purchased from the company. Bovine serum albumin, TMB, HRP labeled anti human IhG, and GolgView I nucleic acid dyes are bought from a biotechnology company in Shanghai.

Protease K, phenol, anhydrous ethanol, rTaq enzyme, carrier like buffer and lysozyme are purchased from a biological science and technology company in Beijing. The main instruments used for the separation of mycobacterium tuberculosis include an inverted microscope, CO2 incubator, pressure steam sterilizer, OLYMPUS microscope, P2 high speed refrigerated centrifuge, biological safety cabinet, and water jacket incubator. Each instrument has been calibrated before use, to meet the use requirements.

Microscopic examination of sputum smear is carried out firstly. The slide preparation, direct smear method, and mirror inspection are done in turn. Use warm water to wash the slides, and pick out the slide that can meet the use requirements. Drop the re red dye solution to completely cover, and repeat for 4-5 times continuous dyeing within 5min. Hydrochloric acid of5% concentration is dropped from the top edge of the sputum film. After the decolorization of 3min, rinse the liquid with running water until purple red is not visible. Dropping compound methylene blue dye for dyeing 1min, rinse again and drain off the excess water. After the completion of the dry, smear can be carried out microscopic examination. In the light blue background, red appears in acid fast bacilli, and blue appears in bacteria or other cells. The production process of sputum smear and microscopic examination are shown in Fig. 2.

The second is to cultivate anti cultivation strains. According to different characters of sputum, 50μL is added into 1.5mL centrifuge tube. Then add 100μL sodium hydroxide solution with 4% concentration, keep oscillation in the oscillator for 2min, and maintain the static for 20min at room temperature after full homogenization. The test tube is rotated, the bacteria liquid is evenly distributed on the inclined plane of the culture medium, and the culture medium is arranged in the incubator, and the culture medium is cultured for 4 weeks at 37 degrees Celsius. Observe the situation of cultivation in the second week, the third week, and the fourth week.

Diagnosis is made by observing the growth of bacteria. If the colony growth is observed, the positive test can be confirmed by microscopic examination. And if the colony growth is not observed, it will continue to be cultured for eighth weeks, and if there is still no colony growth after microscopic examination, it will be determined to be negative.

The process of making clinical isolates of smear is similar to the production process of sputum smear. One single colony grown on a modified Roche medium was

selected in the biological safety cabinet, and a small amount of normal saline is added. Then boil 30min for high pressure sterilization. And the fixation, dyeing, bleaching, dyeing and drying and reexamination of sputum in turn are carried out.

For the strains of the expansion of culture, we must first carry out the preparation of bacterial suspension, with the inoculation ring to take the initial culture 2-3 weeks of mycobacterium tuberculosis, and place it in a sterile test tube. The test tube has two drops of 0.5% concentrations of Tween-80 physiological saline, with the use of glass rod grinding, so that the colonies can be fully dispersed, and then add the normal saline mix after the static set 1h. The supernatant is diluted with normal saline, and when the turbidity is the same as the turbidity of the wheat, the bacterial suspension with 1mg/mL is prepared.

After the bacteria are deposited in the bacterial liquid, the inoculation loop is gradually diluted. By means of scoring method is inoculated into the culture medium containing uniform surface, make it evenly distributed on the culture medium. Culture medium is in the incubator for culture, and the environment is 37 degrees with5% of CO2. And observe in the third day and seventh day, and then observe every other week, and the growth of colonies is observed. When the colony is standing on the inclined surface of the culture medium, the 1/4-2/3 can be stopped and placed in the refrigerator at 4 degrees. The strains are isolated and cultured for many times until the purified strains are obtained.

Study on infectious disease mechanism of mice: The control group and the immune group are set up. In addition, that the mice injected with normal saline is different from that of the immune system, the experiment steps and the environment of the two are consistent. By observing the specific value added transformation of mouse spleen lymphocytes in the two different groups, the cellular immune function of mice is analyzed.

Mycobacterium tuberculosis H37Rv and bacteriophages are provided by a biological research institute in Beijing. Experiment with Bad/c female mice are purchased from the institute of animal science, weight 20g±2, 2-4 weeks. Experimental reagents and consumption type experimental materials are all purchased from a chemical company in Beijing, including H2O2, NaOH, NaHCO3, KH2PO4, ortho benzene diamine, syringe, aluminum foil, gauze, conical flask, Petri dishes, spring clip, iron, commonly used anatomical tools and liquid moving device. The name, type and production of the main equipment and instruments used in the experiment are shown in table 2.

Table 2
Statistics of major equipment used in the experiment

S. N.	Instrument name	Model	Manufacturer
1	Optical microscope	SFC-18	Motic
2	Refrigerator		Qingdao Haier Corporation
3	Carbon dioxide incubator	INCUBATOR	SELECTA
4	Analytical balance	JJ500	Changshou City Shuangjie testing instrument factory
5	Digital display air drying box	HG-907385S-Ⅲ	Shanghai CIMO Instrument Manufacturing Co. Ltd.
6	Microwave Oven	MZ-2270EGC	Qingdao Haier Corporation
7	Electric heating constant temperature incubator	HH.811.360-BS-2	Shanghai Yuejin Medical Instrument Factory
8	Enzyme linked immunosorbent assay	DG5032	Nanjing East China Electronics Co., Ltd.
9	Low temperature refrigerated centrifuge	PK131R	Thermo company
10	Inverted microscope	TS-100	Nikon
11	Super clean working table	SN-CT-IFD	Shanghai Bo Xun Industrial Co. Ltd.
12	High speed centrifuge	TGL-16G	AnKe
13	A magnetic stirrer	85-I	Changzhou Huapu Instrument Co. Ltd.
14	Digital display thermostatic bath	SHA-B	Changzhou Huapu Instrument Co. Ltd.
15	Vertical pressure steam sterilizing pot	YXQ-LS	Shanghai Bo Xun Industrial Co., Ltd.

Firstly, the configuration of ConA and MTT reagent is carried out. 10mgConA and 250mgMTT are dissolved in PBS with the concentration of 0.01mol/L, and PH environment is 7.4. The solvent configured is done with filtration and sterilization, and then stored at 4 degrees Celsius. Other reagents are configured in a similar way, and are stored in the same environment.

The bacteria are diluted to 1mg/ml with normal saline, and the bacteria concentration is adjusted to 3×107/ml under microscope. The experimental group uses in vivo inoculation method, namely in the mouse tail root with a multi-point injection syringe, injection amount of 0.1ml liquid, so that it becomes into a ball. In the second and fourth week respectively, make the plus, and if necessary, make the plus in fifth week. After 2 weeks, collect the two groups of mice and cut the tails, from the root of the tail to tail tip passed slowly. Collect blood and add it in the 1.5ml centrifuge tube, and the blood is placed at 4 degrees Celsius in the environment for 30min. After 30min centrifugation with4000rpm, the supernatant is used as the serum.

12 weeks later, when the serum anti PPDIgG antibody of the immunized mice reaches a certain level, they are put to death and then soaked in the alcohol with the concentration of 75% for 30s. On the operating table, the mouse peritoneal membrane is opened and the spleen is taken out and placed in the culture medium of RPMI-1640 (containing 10% serum). The splenic lymphocytes are extracted after crushing of spleen. Then according to the phage random peptide library titer, they are diluted, with the dilution concentration of 106, 109, and 10^{12}/ml. At the same time, the control group is set up, and with the concentration of 20μg/ml, 2.5μg/ml ConA and PPD for comparison. In the environment of 37 degrees in the carbon dioxide incubator, they are cultured for 48h or so, and then continuously cultured for4h with the addition of MTT. Finally, the absorbance value of the enzyme is measured using Microplate Reader, and the stimulation index is calculated by the value.

Analysis and discussion of the results

Experimental result: Microscopic examination of sputum shows that no acid-fast bacilli are found in 300 different visions of consecutive microscope. In the detection of sputum samples, there are 167 negative bacilli. Judgment standard of sputum smear results is shown in the following table.

Table 3
Criteria for judging results of microscopic examination

S.N.	Consecutive observations	The number of acid fast bacilli	According to the results of the determination of the number of bacteria
1	300	1-2	Suspicious (+)
2	100	3-9	Masculine (1+)
3	10	1-9	Masculine (2+)
4	Each	1-9	Masculine (3+)
5	Each	>10	Masculine (4+)

The results of microscopic examination show that mycobacterium tuberculosis is slender, few are completely red cell, most of them show nodular, cell dispersion, and longitudinal cords. When the time is too long in the medium of mycobacterium tuberculosis, it is easy to change, and the bacteria will break and form the short and spherical particles. The test results have 64 samples of sputum, and microscopy positive rate is 29.01%. The 64 positive samples are isolated from sputum, and colony growth is generally visible within 2-8 days. After 4 weeks of training, the colonies are granular, nodular, opaque white, rough surface, cauliflower. Isolated strains are isolated in sputum as raw material, and the obtained microscopic examination results are consistent with the results of sputum microscopic examination by the prosecution. From the microscopic pictures, it can be seen that some strains show the positive results but not the dispersion of mycobacterium tuberculosis, and some strains show the positive results with the dispersion of mycobacterium tuberculosis. Bacterial liquid is not completely dispersed, so that the strains are clustered together. The strains obtained using sputum for cultivation are as raw materials. Generally, the colony growth is visible in 2-4 weeks, and colonies are particles, nodules, opaque, rough surface.

DO values are detected by enzyme linked immunosorbent assay, finally the level of anti PDD antibody in serum of immunized mice is as follows.

It can be seen from the test results that there is significant difference between the immune group in the second week, fourth week and sixth week. In the control group, the difference is not significant, and the difference between the control group and the immune group is significant.

In the fourth week, the antibody titer is 1:800, and it is 1:3200 in the sixth week.

By displaying the cell count, in the spleen lymph suspension, the number of cells is 1.2×10^7 /mL, the number of cells after the nylon wool column is 3.6×10^6/mL, and the

recovery rate of T lymphocytes is 30%. In 4% blue solution and T lymphocyte suspension (1:1), after 5min, the dead cells are light blue, and the living cells are not stained. After calculation, the ratio of living cells is 93%.

The mouse lymphocyte transformation results show that, PPD, ConA and other phage random peptide library can stimulate the T lymphocytes in mice. In accordance with the titer of 1, 2, 3, from the statistical analysis of the results of the control group and the immune group, it can be seen (see Table 5) that the maximum stimulation index of the phage random peptide library of Mycobacterium tuberculosis is 3.04 in the experimental group, and the calculated value of the immune group is generally larger than that of the normal group, which fully explains that the mice have produced anti TB cell immunity.

Table 4
Serum anti PPD antibody level in immunized mice (x±s, n=15)

Time (weeks)	2	4	6
Saline group	0.061	0.068	0.065
H37Rv immune group	0.081	0.213	0.253

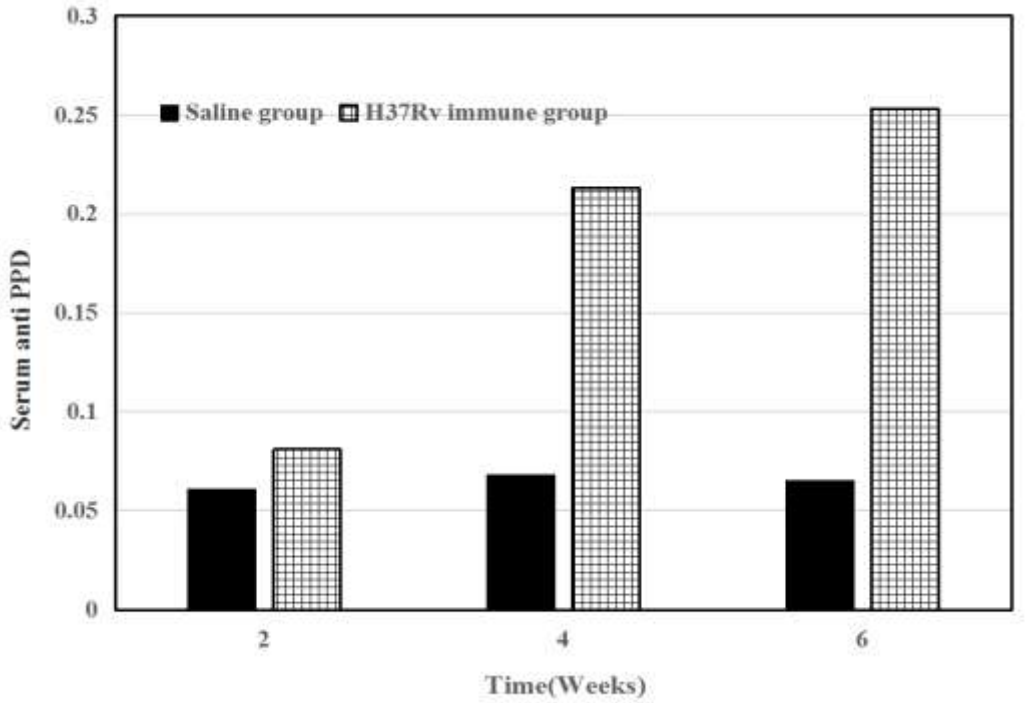

Fig. 3: Immune mouse serum antibody PDD antibody level

Table 5
The specific proliferation and transformation of spleen lymphocytes in mice

Different grouping		Control group	Immune group
PPD		1.55	1.81
ConA		1.97	2.53
Phage random peptide library	Titer 1	1.69	1.88
	Titer 2	1.48	1.71
	Titer 3	1.31	1.56
Mycobacterium tuberculosis phage random peptide library	Titer 1	2.04	3.04
	Titer 2	1.86	2.69
	Titer 3	1.69	2.18
Fourth round phage random peptide library	Titer 1	2.00	2.41
	Titer 2	1.77	2.05
	Titer 3	1.41	1.63

Analysis and discussion: Since the first use of ELISA in patients with serum anti PPD body in 70s and 80s of the last century, the clinical application of the enzyme linked immunosorbent assay is more and more. Chinese scholars have made a deep study on the detection of serum PPD antibody, which has a certain application value. At the same time, the best experimental conditions are determined, and the concentration of PPD is 20μg/ml. In order to ensure the separation efficiency of T lymphocytes and reduce the effects of interference factors on the reliability of experiment, the nylon wool column method for the injection of Mycobacterium H37Rv in mice is selected in this study. PDD is determined, and flow rate of cell suspension is controlled to reduce the pollution of other lymphocytes, so as to improve the purity of T cells, the ratio of living cells and the rate of recovery.

Through the experiment, it can be seen that the recovery rate of T cells is 30%, and the ratio of living cells is 93%. The results show that the ratio of living cells is high. The method of sputum separation culture used in this paper is convenient with low cost, and reliable results can be obtained. The positive rate of bacterial growth could be improved by the re culture of the growing colonies, and the colony growth could be seen in 2-4 weeks, and the results were confirmed by microscopic examination.

In the previous study, 8 week experiment using H37Ra mice immunized with mycobacterium tuberculosis is made to detect the specific cellular immune response level. The results show that there will be a significantly proliferation in vitro after PPD stimulation, and the calculated stimulation value is 2.1. The proliferation and transformation of lymphocytes is one of the indicators for the observation of the immune function of the organism. Different subsets of lymphocytes can make the sensitivity difference because of the different stimuli, and the T lymphocytes of the mouse source have a strong sensitivity to ConA.

Through this experiment, it can be seen that after immunization with mycobacterium tuberculosis, the effect of ConA, PPD and other different stimuli is obvious during the induction of lymphocyte proliferation, and the maximum stimulation index is 3.04. Further immunization study of mice with mycobacterium tuberculosis is conducted, through the analysis of the effect of different stimuli on the proliferation of lymphocytes, and the stimulation index is used to observe the formation of anti-tuberculosis cell immunity in mice. In comparison with the results of the control group and the immune group, we can see that, compared to the control group, the stimulation value of the immune group is larger.

Conclusion

The infection mechanism of mycobacterium tuberculosis and its survival condition in vivo can be studied by using the isolation of T cell concentration after injection of mycobacterium. Through the separation experiments of the sputum of patients with suspected tuberculosis provided in a hospital, the growth and morphology of bacteria in sputum culture and purification can be seen clearly, and the preliminary diagnosis of tuberculosis can be accurately carried out with the method of staining. For experimental study on the mechanism of infectious disease in mice, immune control group and immune experimental group with H37Rv mycobacterium are set up.

The results show that the antibody titers is 1:800, 1:3200, respectively, in the fourth week and the sixth week. By using nylon wool column method, the recovery of T cells can be successfully recovered, the recovery rate of T cells is 30%, and the percentage of living cells is 93%. In the further mouse immunization test, the lymphocyte proliferation and transformation of nuclear H37Ra immune mice under different stimulus conditions are analyzed by the stimulation value. The experimental results show that the stimulation value of immune group is larger, which indicates that the cellular immunity against mycobacterium tuberculosis has produced in the body of mouse, and the maximum calculated stimulation index is up to 3.04.

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