Establishment of Microbial Limit Test Methods for Two Hospital Preparations

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Abstract [**Objectives**] This study was conducted to establish microbial limit test methods for traditional Chinese medicine preparations Yunpi Granules and Bupi Qiangli Paste. [**Methods**] According to General Rules of Part IV of *Chinese Pharmacopoeia*, applicability tests were conducted on microbial limit test methods for the above two traditional Chinese medicine preparations by the plate method. [**Results**] The established methods showed recovery values in the range of 0.5 -2.0 for both experimental strains, and the control bacteria could be detected in the experimental group, but not in the negative control group. [**Conclusions**] The microbial limit test methods were reliable for the two traditional Chinese medicine preparations and could be used for quality control.

Key words Yunpi Granules; Bupi Qiangli Paste; Microbial limit test; Method applicability test

Yunpi Granules and Bupi Qiangli Paste are both prepared based on clinical prescriptions of clinical doctors from the Second Affiliated Hospital of the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine by traditional processes. With the increasing development of drug dosage forms and the continuous improvement of drug quality control standards, accurate and effective microbial limit tests of drugs has become an important topic in the pharmaceutical field. Microbial limit test methods of drugs was first included in the 1995 edition of *Chinese Pharmacopoeia*, and the verification of microbial test methods was added in the 2005 edition, and to the method applicability tests[1] in the 2020 edition of *Chinese Pharmacopoeia*, an important indicator runs through drug quality control, *i. e.*, traditional Chinese patent medicines and simple preparations are compound preparations and contain many herbs, of which many single medicinal herbs have bacteriostatic or antibacterial effects, which inhibit the growth of microorganisms. When testing by conventional methods, there are often cases of abnormal or missing inspection, which cannot accurately reflect the inspection results. In this study, applicability tests were conducted on the testing methods of two traditional Chinese medicine preparations, and a feasible microbial limit test method was established for each. This study not only provides a scientific basis for microbial limit testing, but also improves the detection rates of microorganisms contaminated by the drugs themselves.

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Materials and Methods

Experimental materials

Samples Yunpi Granules, specification: 5 g/bag, batch numbers: 20200801, 20200802, 20200803, and Bupi Qiangli Paste, specification: 120 g/bottle, batch numbers: 20200401, 20200402, 20200403, provided by the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine.

Culture media and diluents pH 7.0 sodium chloride-peptone buffer (batch number 1074152), trypticase soy broth (TSB) (batch number 1075741), trypticase soy agar (TSA) (batch number 1090991), sabouraud dextrose broth (SDB) (batch number 1076161), sabouraud dextrose agar (SDA) (batch number 1090061), MacConkey broth medium (batch number 1075651), and MacConkey agar medium (batch number 1076291), all produced by Guangdong Huankai Biology Co., Ltd.

Strains *Pseudomonas aeruginosa* [CMCC(B)10104], *Staphylococcus aureus* [CMCC(B)26003], *Bacillus subtilis* [CMCC(B)63501], *Candida albicans* [CMCC(F)98001], *Aspergillus niger* [CMCC(F)98003], and *Escherichia coli* [CMCC(B)44102], and *Salmonella paratyphi B* (CMCC(B)50094), all produced by Guangdong Huankai Biology Co., Ltd.

Instruments Electronic balance (model TP-320C, Xiangyi Balance Instrument Equipment); electrothermal thermostatic water bath (model BSG-24, Shanghai Yiheng Scientific Instrument Co., Ltd.); electrothermal thermostatic incubator (model DHP-9162, Shanghai Yiheng Scientific Instrument Co., Ltd.); biosafety cabinet (model HR30-II A2, Qingdao Haier Special Electrical Appliance Co., Ltd.); microbe tester (model JPX-2010, Shanghai Bingyue Electronic Instrument Co., Ltd.).

Experimental methods

Preparation of microbial liquids The microbial liquids were prepared according to the requirements of Part IV of *Chinese Pharmacopoeia* (2020 edition), with reference to the method reported by Liu *et al.*[2-5]. *S. aureus*, *B. subtilis*, *P. aeruginosa* and *C. albicans* were prepared using sodium chloride-peptone buffer

(pH 7.0) into bacterial suspensions with bacterial concentrations not higher than 10 000 and 1 000 cfu/ml, respectively. *E. coli* was prepared into a bacterial suspension with a bacterial concentration not higher than 1 000 cfu/ml. *A. niger* was prepared into fungal suspensions with spore concentrations not higher than 10 000 and 1 000 cfu/ml, respectively.

Preparation of test liquids A certain amount of each tested sample (10 g) was added with TSB to 100 ml, forming a 1 ∶ 10 test liquid after shaking well. In order to screen an effective method for eliminating the antimicrobial activity, the 1 ∶ 10 test liquid was also diluted to 1 ∶ 20 and 1 ∶ 40 test liquids for later use.

Strain recovery

Recovery (%) = (Average number of colonies in the experimental group − Average number of colonies in the control group of test liquid)/Average number of colonies in the control group of microbial liquid × 100

A recovery value in the range of 0.5 − 2.0 indicated that the method was feasible[5].

Applicability of microbial counting method

(i) Experimental group. Plate method: Seven portions (10 ml each) of each of test liquids 1 ∶ 10, 1 ∶ 20 and 1 ∶ 40 were added into sterile glass tubes, respectively. Among them, five portions were added with 0.1 ml of bacterial suspensions under item "Preparation of microbial liquids" (five strains with a concentration no higher than 10 000 cfu/ml corresponding to aerobic microbe counting), respectively, and the remaining two portions were added with 0.1 ml of *C. albicans* and *A. niger* corresponding to mold and yeast counting, respectively. The obtained liquids were shaken well to ensure the final concentrations of microbes equal to or lower than 100 cfu/ml. Next, 1 ml of the seven test liquids were, respectively, added into 90 mm sterile culture dishes, into each of which 20 ml of culture medium (TSA for aerobic microbe counting plates, SDA for mold and yeast counting plates) melted at about 45 ℃ was added, and the obtained liquids were quickly mixed, cooled and solidified.

(ii) Control group of microbial liquid. The test liquids were replaced with TSB, and the operation accorded to the above method under "Experimental group".

(iii) Control group of test liquid. The microbial liquids were replaced with TSB, and the operation accorded to the above method under "Experimental group".

(iv) Negative group. The test liquids were replaced with TSB, and no microbial liquids were added, and the operation accorded to the above method under "Experimental group".

Applicability test for control bacteria

(i) Experimental group. First, 10 ml of a 1 ∶ 10 test liquid prepared under item "Preparation of test liquids" was inoculated into 100 ml of trypticase soy broth, respectively, and 0.1 ml of prepared bacterial suspensions of *E. coli* and *S. aureus* were added, respectively. After mixing well, the strains were cultured at 34 ℃ for 18 − 24 h. Next, 1 ml of each above culture was inoculated into 100 ml of MacConkey liquid medium, and cultured at 44 ℃ for 48 h. The MacConkey liquid cultures were streak-inoculated on MacConkey agar medium plates, and cultured at 34 ℃ for 24 − 72 h.

(ii) Test liquid group. Ten milliliter of a 1 ∶ 10 test liquid prepared under item "Preparation of test liquids" was inoculated into 100 ml of trypticase soy broth, and the culture conditions and examination method were the same as those of the experimental group.

(iii) Negative group. Ten milliliter of sterile sodium chloride-peptone buffer (pH 7.0) was inoculated into 100 ml of trypticase soy broth, and the culture conditions and examination method were the same as those of the experimental group.

Results and Analysis

Comparison of test liquid dilution counting methods

The recovery data of aerobic bacterial counts (two significant digits) using the test liquid dilution method are shown in Table 1. According to the pre-experiment results, when the test liquids of Yunpi Granules were 1 ∶ 10, 1 ∶ 20, and 1 ∶ 40, the recovery values of the five experimental groups for five types of microbes were all within the range of 0.5 − 2.0. From the recovery values, when the test liquid was 1 ∶ 10, the recovery of *S. aureus* was 0.65, close to 0.5, indicating that the preparation had weak antibacterial effect on *S. aureus*. When the test liquids of Bupi Qiangli Paste was 1 ∶ 20 and 1 ∶ 40, the recovery values of the five experimental microbes in the seven experimental groups were all within the range of 0.5 − 2.0. The results showed that when the test liquid was 1 ∶ 10, Bupi Qiangli Paste had a strong inhibitory effect on the growth of *S. aureus*, and its recovery was far lower than the lower limit of 0.5. When the test liquid was diluted to 1 ∶ 20 and 1 ∶ 40, the antibacterial activity of the preparation could be significantly eliminated.

Table 1 Results of pre-experiment on applicability of plate dilution method for total aerobic microbe counting

Sample	Dilution ratio	Batch number	Aerobic microbe counting					Mold and yeast counting	
			P. aeruginosa	*S. aureus*	*B. subtilis*	*C. albicans*	*A. niger*	*C. albicans*	*A. niger*
Yunpi Granules	1 ∶ 10	20200801	0.86	0.65	0.93	0.81	0.91	0.83	1.10
	1 ∶ 20	20200802	0.84	0.69	0.98	0.85	0.90	0.90	1.10
	1 ∶ 40	20200803	0.79	0.79	1.00	0.91	0.95	0.98	1.20
Bupi Qiangli Paste	1 ∶ 10	20200401	0.82	0.08	0.81	0.86	0.96	0.97	0.97
	1 ∶ 20	20200402	0.92	0.81	0.92	0.96	0.91	0.98	0.91
	1 ∶ 40	20200403	0.95	0.83	0.95	1.00	0.84	1.00	0.88

Results of applicability test for control bacteria

According to the treatment method under "Applicability test for control bacteria", the experimental group of the above method was positive, and the colonies on the plates were isolated, cultured and subjected to Gram-staining microscopy, confirming that the strain was *E. coli*. Both the test liquid group and the negative control group were tested to be negative. Therefore, the control bacteria examination of the products could be operated by the routine method. The results are shown in Table 2.

Table 2 Results of applicability test on the examination method for control bacteria *E. coli* ($n=3$)

Sample	Treatment	TSB	MacConkey broth medium	MacConkey agar medium
Yunpi Granules	Experiment group	+	+	+
	Test liquid group	−	−	−
	Negative group	−	−	−
Bupi Qiangli Paste	Experiment group	+	+	+
	Test liquid group	−	−	−
	Negative group	−	−	−

+ stands for the growth of bacterial colonies, and − stands for no growth of colonies.

Conclusions and Discussion

Yunpi Granules and Bupi Qiangli Paste are both non-sterile oral Chinese herb preparations that do not contain the original medicinal powder. Yunpi Granules are composed of nine medicinal herbs, including bran fried Rhizoma Atractylodis, earth fried Rhizoma Atractylodis Macrocephalae, Poria, Semen Coicis, fried Fructus Aurantii and Rhizoma Dioscoreae. Bupi Qiangli Paste is composed of ten medicinal herbs, including Radix Astragali, Prepared common monkshood daughter root, Herba Epimedii, Herba Cynomorii, and Radix Codonopsis. The two preparations had different preparation methods, but the microbial examination methods tend to be consistent, both using the plate method. Due to the strong antibacterial effect of Bupi Qiangli Paste on *S. aureus*, the applicability of the culture medium dilution method for total aerobic microbe counting was studied. Based on the experimental data, following conclusions could be drawn: Yunpi Granules did not show any obvious antibacterial components during the method applicability counting test; and when conducting the applicability test of the method, Bupi Qiangli Paste had antibacterial active ingredients and showed strong antibacterial ability against *S. aureus*, of which the recovery value did not reach above 0.5, so it was necessary to adopt the test liquid dilution method. Finally, the recovery values in aerobic microbe counting using the 1∶20 test liquid were within the range of 0.5 – 2.0.

In the 2020 edition of *Chinese Pharmacopoeia*, the main ways to reduce the bacteriostatic activity in the microbe counting method were increasing the volume of diluent or culture medium used, adding a neutralizer or an inactivator in an appropriate amount, using membrane filtration method or using the combination of the above three methods[6]. In the process of applicability tests for microbes, when a sample exhibits antibacterial effects, we first consider increasing the volume of diluent or culture medium used to eliminate the antibacterial components, and if the antibacterial components could not be completely eliminated, the method of adding neutralizers or inactivators will then be considered. If there is no suitable method to eliminate the antibacterial effect in a test sample and the recovery test fails, the membrane filtration method will be considered to verify and compare the method applicability for the preparations. Through the applicability tests on various microbial limit test methods for traditional Chinese medicine preparations, the microporous filter membrane in the membrane filtration method can filter soluble antibacterial components and effectively intercept bacteria, and contamination bacteria in preparations could be successfully detected thereby. The method is fast, simple, and has high accuracy. The membrane filtration method for microbial limit testing of drugs can effectively remove antibacterial components from drugs, weaken antibacterial effects, and effectively avoid interference from the operating environment and the ability of inspectors, thus ensuring the accuracy of drug testing as much as possible[7]. Although the culture medium dilution method can dilute the antibacterial components in drugs, it cannot completely eliminate them, especially in traditional Chinese medicine compound preparations, which have complex and diverse components, and contain one or more antibacterial components that may be detrimental to the growth of experimental bacteria, thereby affecting the accuracy of detection. Moreover, the antibacterial ability of traditional Chinese medicine compound preparations produced from different batches of medicinal materials varies. We conducted method applicability tests using three batches of finished preparations to meet the reproducibility of microbial limit test methods for traditional Chinese medicine preparations and effectively control drug quality.

The 2015 edition of pharmacopoeia changed the "method validation" of microorganisms to "method applicability", with a more flexible focus on sample pre-treatment, selective cultivation of microorganisms, and identification confirmation, providing space for innovative development of testing technology. Factors such as experimental conditions and test product characteristics are evaluated as a whole system to control the impact of analytical conditions on various factors, so that testing activities can achieve the expected goals, which is conducive to the transformation from final product inspection to process control for drug quality. On this basis, in this study, combining with the complexity of traditional Chinese medicine preparations, we tested the total aerobic microbe count in Yunpi Granules by conventional methods and that in the 1∶20 test liquid of Bupi Qiangli Paste by the culture medium dilution method, and examined the control bacteria *E. coli* by conventional methods, which could detect positive bacteria under specified cultivation conditions.

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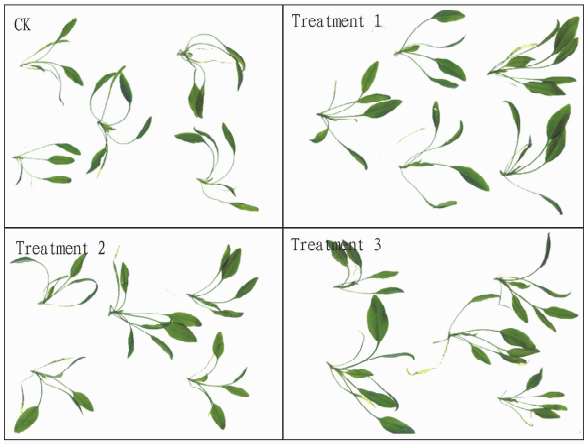

Fig. 1 Morphology of spinach leaves in treatments with different application rates of bioorganic fertilizers

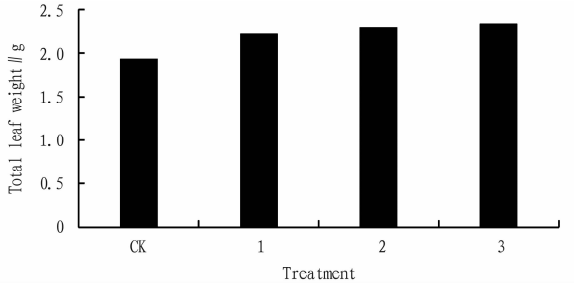

Fig. 2 Effects of different application rates of organic fertilizer on total leaf weight of spinach plant

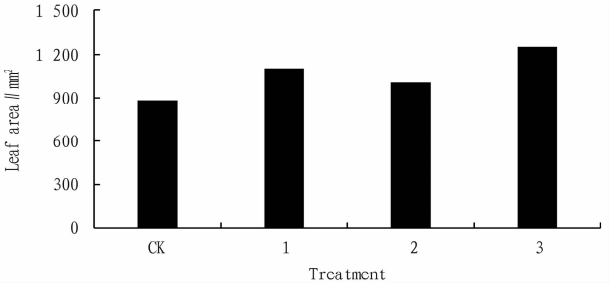

Fig. 3 Effects of different application rates of organic fertilizer on leaf area of spinach plants

the application rate of bioorganic fertilizer increasing among treatments, while the leaf area fluctuated with the increase of bioorganic fertilizer concentration among various treatments. The average root volumes and average diameters of treatments applied with bioorganic fertilizer at different rates were all higher than those in the CK, but the average root length showed a different trend. The average root length was higher in the CK than in various bioorganic fertilizer treatments in the early growth period, but the differences of various treatments from the CK gradually decreased in the middle period, and in the later period, the root length of spinach treated with organic fertilizer gradually exceeded the CK. The study on the effects of bioorganic fertilizer at different application rates on spinach leaf area, root growth and other indexes provides data support and a theoretical basis for the promotion and application of bioorganic fertilizer, as well as a reference basis for promoting fertilizer reduction and efficiency improvement.

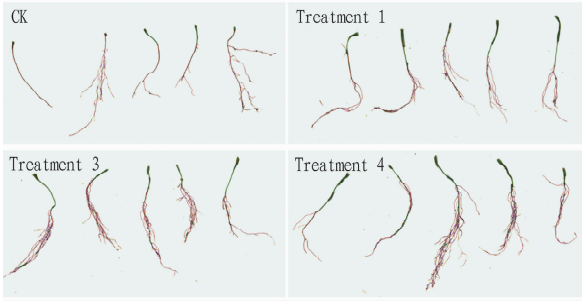

Fig. 4 Treatment of spinach root morphology with different application rates of bioorganic fertilizer

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