2) The correlation between the design of pharmaceutical water equipment and pharmaceutical water quality
3) Control methods for pharmaceutical water equipment (including methods of sanitization, sterilization, and disinfection)
4) Test methods and control limits for pharmaceutical water
5) Characteristics of microorganisms in the facility and water system equipment (e.g., concentrations of residual chlorine, biofilms, endotoxins, resistance to sanitation)
6) Sampling procedures and related precautions
7) Validation, change control, and deviation control for pharmaceutical water equipment

A2.5 Maintenance of Pharmaceutical Water Equipment

A preventative maintenance program should be established for the preservation of well-controlled water system operating conditions. The program should include, but not be limited to, the following items:

1) Procedures for operating the water system
2) Programs for monitoring operating conditions and key water quality characteristics
3) A schedule of periodic sanitization
4) Preventative maintenance of each component of the system and calibration of instruments
5) Change control of mechanical systems and operating conditions
6) Procedures for temporary stoppage and resumption of the membrane filtration water treatment system

A2.6 Change Control

When water system equipment is remodeled or expanded, or system operation procedures are modified, the potential impact of these changes on the water system itself should be evaluated. The necessity for system reevaluation should also be determined, and, if judged to be necessary, reevaluation should be performed as required according to the validation protocols, in a manner similar to that performed at the time of system installation. The procedure for the control of these changes should be established as part of the validation of the maintenance program.

A2.7 Deviation Control

Procedures for the water system equipment to be taken when parameters exceed the alert or action level should be established and implemented according to appropriate deviation control SOPs. Deviation control should include, but not be limited to, the following items:

1) Reporting procedure
2) Resampling and retesting procedure
3) Handling procedures for deviated pharmaceutical water and for in-process or finished products manufactured using such deviated pharmaceutical water
4) Preventive measures
5) Corrective measures
6) Review of monitoring procedures and alert and action levels

A3. Biohazard Control

A3.1 Biosafety levels

Biosafety levels (BSL) should be established depending on the pathogenicity of microorganisms used for the manufacture of individual biological drug products. Production facility and equipment should be suitable for BSLs, which are classified as described below. After inactivation or removal of the microorganisms, processes may be similar to those for non-biological drug products. Facilities and equipment that meet higher BSL requirements, as determined by appropriate procedures, can be considered to have met lower BSLs without testing for them.

1) BSL1: The risk of potential exposure of the operator who handles microorganisms and those in the surrounding environment to infectious agents is not evident or is minimal (examples: attenuated measles virus, attenuated rubella virus, attenuated mumps virus, attenuated chickenpox virus, and attenuated poliomyelitis virus, etc.).

2) BSL2: The risk of potential exposure of the operator who handles microorganisms and those in the surrounding environment to infectious agents is moderate and low, respectively (examples: *Bordetella pertussis, Corynebacterium diphtheriae, Clostridium tetani, Vibrio cholerae,* influenza virus, Japanese encephalitis virus, etc.)

3) BSL3: The risk of potential exposure of the operator who handles microorganisms and those in the surrounding environment to infectious agents is high and low, respectively (examples: *Bacillus anthracis*, *Yersinia pestis*, new influenza virus, etc.)

4) BSL4: The risk of potential exposure of both the operator who handles microorganisms and those in the surrounding environment to infectious agents is high (in practice, this level is never applied to medicinal product manufacturing facilities).

A3.2 Controlled Areas

Areas where handling of microorganisms are regulated depending on the BSLs for safety purposes are called "controlled areas." The entry and exit doors of controlled areas should be designated as such with readily visible markings. Also, biohazard label(s) should be posted, indicating the microbes to be handled, BSL, name of the person responsible for the controlled area, emergency contact information, etc., as appropriate.

A3.3 General Requirements for BSL1 Facilities

1) There are no specific requirements for facilities and equipment applicable to BSL1 facilities.
2) Waste materials contaminated with microorganisms including carcasses ("waste materials" include carcasses, hereafter) should be disposed of as described below.

   Waste materials should be sterilized by appropriate means (e.g., chemical agents or heat) and either transferred out of the BSL1 facility, or placed in a shatterproof and leakproof container whose exterior is appropriately disinfected, and transferred out of the BSL1 facility. The waste material will then be incinerated. It is acceptable practice to contract out incineration of the waste if the waste materials are sterilized beforehand.

A3.4 General Requirements for BSL2 Facilities

1) Any operations that may generate aerosolized microorganisms should be conducted in a closed apparatus equipped with a HEPA filter, a Class IIA or higher safety cabinet (or equivalent). In addition, air exhausted from such an apparatus or system should be cleaned so as to completely eliminate aerosolized microorganisms.
2) If there is a risk of aerosolized microorganism generation in the BSL2 facility, exhaust air should be cleaned using a HEPA filter before exhaustion.
3) Waste materials contaminated with microorganisms should be disposed of using one of the procedures listed below. It is an acceptable practice to contract out incineration of the waste if waste materials are sterilized beforehand.

(A) Waste materials should be sterilized appropriately (e.g., chemical agents or heat), transferred out of the BSL2 facility, and incinerated.

(B) Waste materials should be placed in shatterproof and leakproof containers whose exterior is appropriately disinfected, transferred out the BSL2 facility, and incinerated.

(C) Waste materials should be directly transferred from the BSL2 facility to either an incinerator or to a sterilizer for subsequent incineration using an appropriately managed closed system.

4) Waste fluids containing microorganisms, or those that come into direct contact with microorganisms, should be disposed of after appropriate treatment with chemical agents or heat sterilization in a tank, etc. placed inside or outside the controlled area.

5) Operation areas or rooms for handling smallpox pathogen, acute poliomyelitis pathogen, spore-forming pathogens, or mycobacterium tuberculosis should be equipped with a dedicated HVAC system. Suitable label(s) should be placed on individual apparatuses and devices, and the use of this equipment should also be exclusive. An independent air handling system as well as the air supply and exhaust port should be equipped with a HEPA filter.

A3.5 General Requirements for BSL3 Facilities

1) Controlled areas designated for the handling of microorganisms that require BSL3 containment should be structurally separated from other areas.

2) Restricted entry into the BSL3 facility should be imposed by posting notices indicating the restrictions and by establishing the procedures for gaining admittance, etc. Additionally, there should be suitable means of physical restriction installed, such as a security door.

3) The ceiling, walls, and floor of the controlled areas in the BSL3 facility should be smooth-surfaced, crack-free, non-dust- or debris-shedding, and resistant to chemicals or other types of disinfectants.

4) If a pressure difference is to be maintained against adjacent areas or rooms, the layout of the BSL3 facility processing area or room should include an anteroom. The anteroom should have airtight interlocking double door designed so that both doors cannot be open at the same time.

5) Personnel movement and workflow lines should be unidirectional whenever feasible in order to minimize cross-contamination.

6) Facilities and equipment in the BSL3 facility should be designed to prevent pathogens from being transferred to other areas during culture or storage. Separately, effective

disinfecting apparatuses or devices should be installed as countermeasures against potential pathogen leaks.

7) Faucets in rest rooms and washing sinks, etc. should be automatic, elbow-handled, or pedal operated in order to prevent cross-contamination.

8) Work areas in the BSL3 facility should have enough space to minimize the chance of accidents occurring during processing.

9) The pressure difference of air between rooms where a pressure difference is set should be monitored using a differential manometer.

10) The HVAC system should be designed to facilitate sterilization with formalin and other gases.

11) If any operation carries a risk of generating aerosolized microorganisms, the operation should be conducted in a safety cabinet (Class IIB or higher safety level) equipped with a HEPA filter or other closed and contained systems of a Class IIB or higher safety level. Additionally, the air from such cabinet or equipment shall be exhausted outside of the facility after passing through a HEPA filter.

12) Air in the BSL3 facility should be filtered and exhausted through an independent HVAC system equipped with a HEPA filter. Air should not be circulated within an area.

13) The BSL3 facility should be capable of physically containing microorganisms within the controlled area under any contingent circumstances, such as shut-down of the HVAC system.

14) An emergency power supply should be available to maintain continuous operation of the HVAC system in the event of a power failure.

15) The drain system should be mounted with a device to prevent backflow.

16) Waste fluids containing microorganisms or those that come into direct contact with microorganisms should be sterilized by appropriate treatment with chemical agents or heat and then be disposed of in a tank or other appropriate receptacle placed either inside or outside the controlled area

17) Waste materials contaminated with microorganisms should be disposed of using one of the following procedures:

    (A) Waste materials should be treated by appropriate means of sterilization, e.g., chemical agents or heat, transferred out the BSL3 facility, and incinerated within the manufacturing facility.

(B) Waste materials should be directly transferred (using an appropriately managed closed system) to an incinerator outside of the BSL3 facility, and incinerated within the manufacturing facility.

18) To prevent infection, operators should wear infectious pathogen-resistant protective gear to prevent infection, and use proper gowning and degowning procedures. The gear must be of a protection level suitable for the situation in which it is worn (e.g., a pressurized protective garment).

A3.6 Emergency Safety Measures

The following emergency procedures should be established and documented in case of a potential aerosolized microorganism leak, leak of culture media, fire, or natural disaster:

1) Emergency and first aid for injured personnel
2) Decontamination procedures
3) Emergency communication networks

A3.7 Personnel Training

Biosafety training programs should include the following topics:

1) Characteristics of microorganisms to be handled in the biologically controlled area (e.g., BSL, mode of infection)
2) Procedures for entry into and exit from the controlled area
3) Handling and operating procedures for equipment and devices in the controlled area
4) Procedures for disposal of infectious waste materials
5) Emergency safety measures

A4. Chemical Hazard Control

A4.1 Chemical Hazard Levels

As drugs are pharmacologically active substances, it is critical to routinely control the extent of exposure of personnel to drug components or prepared products during an 8-hour shift.

Example chemical hazard control levels are shown below.

Exposure control level		Level 1	Level 2	Level 3	Level 4	Level 5
Allowable concentration (μg/m3)		1000 – 5000	100 – 1000	1 – 100	≤ 1	Not detected
Pharmacological activity	Daily allowance (mg/day)	> 100	10 – 100	0.1 – 10	< 0.1	< 0.1
Toxicity	Oral (mg/kg)	> 2000	500 – 2000	50 – 500	5 – 50	< 5
		Low toxicity	Low toxicity	Somewhat toxic	Toxic	Highly toxic
	Intravenous (mg/kg)	> 100 Low toxicity		7 – 100 Toxic		< 7 Highly toxic
Other	Carcinogenicity*	–	–	–	2A, 2B	1
	Hypersensitivity	Slight	Slight to moderate	Moderate	Moderate to severe	Severe
Necessity of isolator/barrier system		None	None	Yes	Yes	Yes

* Carcinogenicity assessment by Japan Society of Occupational Health

There is a variety of equipment suitable for the containment of chemical hazards. It is important to select the most suitable equipment understanding the characteristics of the drugs (chemical hazards) to be handled and to establish appropriate levels of hazard control.

A4.2 Calculation of Allowable Exposure Limits

1) Daily allowance

The daily exposure allowance for chemical hazards can be calculated using the following equation:

Estimated no effect value (ENEV) = LD_{50} × 0.0005*

(* 0.0005: Obtained empirically from a comprehensive toxicology database)

Daily allowance = ENEV/Safety factor × body weight (kg)

Safety factor: typically about 100

2) Daily respiratory intake

Theoretical daily respiratory intake can be calculated by using the equation shown below. The daily intake should not exceed the daily allowance.

Daily respiratory intake = Exhalation volume × respiratory frequency per minute × working time (min) × chemical hazard concentration

Exhalation volume: Approximately 2 liters

Respiratory frequency (/min): Approximately 14 breaths

When an isolator is operated under positive pressure, the chemical hazard concentration should be less than 0.0001 during normal operating conditions, assuming a typical isolator leak rate of 0.5%/h.

A4.3 Facility Requirements for Protection Against Chemical Hazards

1) Facilities that handle Level 3 drug substances should be a closed-system (barrier- or isolator-system) facility.
2) Facilities that handle Level 4 drug substances should be a fully closed-system facility.
3) Facilities that handle Level 5 drug substances should be closed-system facilities, where manual operations and human intervention have been eliminated. Operations in a Level 5 facility should be performed by robotic manipulation and/or remote control. The closed system should be maintained under negative pressure relative to the surrounding environment.
4) A barrier system should be installed in rooms of a Class C cleanliness level or higher. Airflow from inside the isolator should not be directed toward personnel.
5) Except for when Level 5 substances are being handled, the inside of the isolator system may be maintained at a positive pressure.
6) The isolator system should be installed in Grade C or higher cleanliness level rooms if the isolator pressure differential is maintained negative to the surrounding environment.

A4.4 General Requirements for Chemical Hazard Protection

1) Restriction of general entry into the manufacturing facility should be imposed by posting a notice indicating the restriction and procedures for gaining admittance, etc. Additionally, there shall be suitable means of physical restriction installed, such as a security door.
2) The ceiling, walls, and floor of the controlled areas in the Level 3 facility should be smooth-surfaced, crack-free, non-dust- or debris-shedding, and resistant to damage from chemicals or other types of disinfectants.
3) If pressure is to be maintained at a higher level than in adjacent areas or rooms, the layout of the Level 3 facility processing area or room should include an anteroom. The anteroom

should have airtight interlocking double door designed so that both doors cannot be open at the same time.

4) Personnel movement and workflow lines should be unidirectional whenever feasible in order to minimize cross-contamination.
5) Faucets in rest rooms and washing sinks, etc. should be automatic, elbow-handled, or pedal operated in order to prevent cross-contamination.
6) Work areas in the facility should have enough space to minimize the chance of accidents occurring during processing.
7) Air supply inlets and exhaust outlets as well as water drains should be equipped with suitable hazard collection and/or inactivation units. The water drainage system should be independent.
8) The HVAC system should be installed as an independent system.
9) Air inlet and outlet devices should be equipped with a HEPA filter, and air should not be circulated within the facility.
10) An emergency power supply should be available to maintain continuous operation of the HVAC system in the event of a power failure.
11) To prevent chemical exposure, operators should wear protective gear, and use proper donning and doffing procedures. The gear must be of a protection level suitable for the situation in which it is worn (e.g., a pressurized protective garment).

A4.5 Personnel Training

1) Chemical hazard training programs should include the following topics:
    a) Toxicity of drug substances to be handled (acute and chronic toxicity)
    b) Procedures for entry into and exit from the controlled area
    c) Handling and operating procedures for equipment and devices in the controlled area.
    d) Procedure for disposal of active waste materials
    e) Emergency safety measures
2) Emergency training should include the following items:
    a) Emergency and first aid care for injured personnel
    b) Decontamination procedure
    c) Emergency communication networks

A5. Tests and Inspections

A5.1 Endotoxins

A5.1.1. General Requirements

1) A risk management system for the prevention of contamination with endotoxin should be employed and implemented adequately for the quality control of raw materials, containers, closures, pharmaceutical water, and equipment for the manufacture of parenteral drug products. The level of control should also be dependent on either the administration route of the parenteral drug product or the risk of contamination of ophthalmic solutions with endotoxins.
2) Equipment and devices used for the manufacture of parenteral drug products should be appropriately controlled to reduce bioburden and, as a result, reduce the endotoxin burden associated with the products. In particular, all surfaces that could come into contact with the intermediate products either before or after filtration sterilization should be carefully monitored and supervised. Equipment and devices should be adequately designed for easily disassembling, assembling, washing, sanitization, and sterilization.
3) Appropriate control tests should be performed on raw materials and pharmaceutical water for the production of parenteral drug products. Control tests should be also performed for raw materials of parenteral drug products by establishing control limits for the test results even if the manufacturing process has endotoxin removal process such as membrane filtration.
4) When purified water is used to wash the equipment, devices, containers, and closures necessary for the manufacture of parenteral drug products, the final rinse water should be of WFI (water-for-injection) quality produced by distillation or ultra filtration. Washing should be performed repeatedly using a sufficient amount of water. The time from washing until sterilization should be kept to the minimum in order to prevent microbial growth.
5) Endotoxin testing should conform to the Established Limits for Bacterial Endotoxins found in the General Information Section of the latest version of the Japanese Pharmacopoeia. The testing procedures shall specify the methodology of inhibition and enhancement tests, determination of non-inhibitory concentrations of substrate determination of minimum amount of substrate required, and maximum valid dilution.
6) When the limulus test is not valid for the detection of endotoxins or endotoxin-like substances, pharmaceutical products should conform to the pyrogen test using a hare, as

described in the General Tests, Processes, and Apparatuses Section of the Japanese Pharmacopoeia.

A5.1.2. Validation

1) When endotoxin is removed by washing, heat treatment, membrane filtration, or adsorption, the endotoxin burden should be measured beforehand, and the post-treatment removal rate should be validated to verify that residual endotoxin levels fall within specified control limits. Endotoxin reduction should be validated using a challenge test under worst-case conditions. The rate of reduction should be at least a 3-log reduction (99.9%). Procedures for periodic revalidation of endotoxin removal should be also established.
2) Endotoxin test should be supported by appropriate method validation. Testing reagents and other materials necessary for endotoxin testing should be appropriately controlled.

A5.2 Insoluble Particulate Matter

A5.2.1. General Requirements

1) Procedures for cleaning containers and closures of parenteral drug products should be established, and insoluble particulate matter derived from the container and closure should be quantitatively evaluated.
2) Procedures for cleaning the product contact surfaces of equipment for parenteral drug manufacture in processes after filter sterilization should be established, and insoluble particulate matter derived from the product contact surfaces of equipment should be quantitatively evaluated.
3) Insoluble particulate matter contained in raw materials and pharmaceutical water to be used for the manufacture of parenteral drug products should be quantitatively evaluated before preparation of the drug solution as well as before and after filter sterilization of the prepared drug solution.
4) The potential impact of volatile substances that could be adsorbed by freeze dried materials during the freeze-drying process should be carefully evaluated when powder to be reconstituted for injection is manufactured by the freeze-dry method.
5) The potential time-dependent and spontaneous formation of insoluble particulate matter through an interaction between the drug solution and the container/closure system, or

aggregation of protein and other high polymeric molecules should be carefully evaluated and verified by long-term stability testing.

6) Parenteral drug products should be inspected for insoluble particulate matter according to the Insoluble Particulate Matter Test for Injectables in the General Tests, Processes, and Apparatuses Section of the Japanese Pharmacopoeia.

A5.2.3. Validation

The instruments used for the insoluble particulate matter test should be validated, and procedures for routine monitoring and control as well as periodic calibration by the supplier using standard particles should be established.

A5.3 Container Integrity

A5.3.1. General Requirements

1) The integrity of the container seals of the sterile drug products should be assured by routine monitoring and in process control or by inspecting 100% of the containers in order to maintain sterility until the time of use. In order to perform this verification, suitably designed stability tests should be conducted to assure the integrity of the container closure system during product shelf life.
2) Test methods for assessing container integrity should be established for individual container closure systems. Possible causes of breaches of integrity include cracks in the container or the loosening of the cap over time caused by changing environmental conditions including temperature variations during storage, vibrations or shocks during the packaging process or handling, and variations in atmospheric pressure during air transportation, etc. Even if integrity is ensured immediately after manufacture, appropriate countermeasures, such as removal of such potential defects by inspection should be established to minimize possible integrity breaches.
3) The degree of sensitivity for the container integrity tests should be specified.

A5.3.2. Validation

1) Validation should be performed to demonstrate the reliability of the container integrity tests employed.

2) Container integrity should be validated by employing challenge tests where such factors are incorporated as the expected temperature variations that would occur during storage, the vibrations and shocks that would be incurred during packaging and transportation, and variations in atmospheric pressure that would occur during air transportation, etc.

A5.4 Visual Inspection

A5.4.1. General Requirements

1) When assurance of the sterility of drug products is found to be dependent on the elimination of containers that exhibit observable integrity failures, the relationship between container integrity and these visual characteristics should be appropriately defined. Standard test procedures for visual inspection should then be established based on Japanese Pharmacopoeia guidelines as listed below.
2) The criteria for visual inspection tests should be specified for each test according to JIS, MIL (Military Standard), or other applicable standards. The limits for foreign matters should be established based on the Foreign Insoluble Matter Test for Injectables in the General Tests, Processes, and Apparatuses Section of the Japanese Pharmacopoeia.
3) When a reference standard is to be prepared and used for the interpretation of results of a visual inspection, the quality of the samples should be evaluated and approved by the quality control unit or other appropriate division.
4) Documentation concerning visual inspection (e.g., SOPs for visual inspection, training SOPs and manuals, and SOPs for testing personnel capabilities) should be prepared and updated as appropriate.
5) SOPs for visual inspection should specify the test methods.

   For example, visual inspection should include, but not be limited to, the following testing conditions:

   - Inspection procedure, inspection pitch, time required for inspection of the individual unit, and the interval of the inspector's rest breaks
   - Inspection benches, inspection conveyers, inspection lamps, inspection tools and instruments (e.g., magnifiers), and inspection posture (e.g., seat)
   - Light intensity over inspection benches, light intensity in the inspection area or room, and color of background plate

   Automatic machines should be subject to, but not limited to, the following testing conditions:

- Models of inspection machines, inspection pitch, time required for inspection of the individual unit
- Assessment methods for the performance of the inspection machines at the beginning and end of the inspection operation, as well as periodic confirmation of the performance using reference standards, etc.
- Calibration

6) Written SOPs should be prepared for the education and training of inspectors. The training should be performed according to the SOPs using reference standards, etc. Training efficiency should be periodically assessed by observing whether inspectors apply the knowledge in practical situations.
7) Procedures for handling cases of unusual and specific findings of defects should be established. Whenever new type defects are found, the quality of such products should be evaluated in detail, the actual character of the defect should be identified, and the cause(s) identified. If the rate of rejected products by visual inspection becomes unacceptably high, the specified rate of rejection should be re-evaluated.

A5.4.2. Validation

1) When inspection is conducted manually by inspectors rather than machines, the efficiency of each inspector should be evaluated using reference standards, etc., to ensure that every inspector's inspection capacity meets a predetermined level. Every inspector's capacity, as well as eyesight, should be assessed periodically.
2) The capacity of automatic inspection machines to inspect the predetermined visual quality of the product and to remove predetermined defects should be validated using reference standards, etc.

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分担研究報告書

USP/EP微生物試験法の評価研究，微生物の迅速検出法の日局導入

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研究要旨：細菌迅速試験法である蛍光活性染色法およびマイクロコロニー法について、技術研修会を開催するとともに、迅速試験法に対するアンケート調査を行った。また、これらの手法に対する操作の習熟度と測定値の個人差の関係について、考察を行った。

A．研究目的

環境微生物学分野における多くの研究により、自然環境中の微生物の大部分は通常の方法では培養困難であることが、明らかとなってきている。これにともない、微生物を培養することなく、迅速・簡便、さらには高精度に定量するための手法が検討されている[1-8]。中でも蛍光試薬を用いて微生物を染色し蛍光顕微鏡で検出・計数する直接計数法は、環境微生物学分野を中心に広く用いられてきている。

今年度は微生物の迅速試験法について、以下を目的とし、研究を実施した。

1．細菌細胞内のエステラーゼ活性を指標として生菌数を迅速・高精度に計数可能な蛍光活性染色法[2]、および細菌の増殖能を指標として生菌数を簡便かつ高精度に測定可能なマイクロコロニー法[3]に関する技術研修会の開催。

2．細菌迅速試験法の技術研修会の参加者に対してアンケート調査を行い、これらの手法の今後の展開に生かす。

3．細菌迅速試験法に対して、操作の習熟度と測定値の個人差の関係について、考察する。

B．研究方法

1．細菌迅速試験法の技術研修会を、平成17年9月1日から2日にかけて、大阪大学大学院薬学研究科・那須研究室で開催した。研修にあたっては、試料として、市販の除菌・殺菌処理をしていないナチュラルミネラルウォーターを用いた。試料中の細菌数を蛍光活性染色法およびマイクロコロニー法で測定した。

2．上記の技術研修会の参加者に対して、①興味をもった手法、②新手法に対するコメント、③難しいと感じられた点について、アンケートを実施した。

3．細菌迅速試験法に対して、操作の習熟度と測定値の個人差の関係について検討するための共同実験を、平成18年2月28日に行った。実験は大阪大学大学院薬学研究科・那須研究室の他、蛍光顕微鏡を有する複数の施設で行った。試料として、標準株（大腸菌）を異な

る菌数で添加した注射用水を用いた（サンプルＡ：10^4 cells/mL、サンプルＢ：10^6 cells/mL、サンプルＣ：0 cells/mL)。各試料中の細菌数を蛍光活性染色法、マイクロコロニー法、平板培養法（混釈法）で測定し、得られた結果を比較した。蛍光活性染色では 6-carboxyfluorescein diacetate と 4',6-diamidino-2-phenyl indole を用いた二重染色法（6CFDA-DAPI 二重染色法）を用いた。計数には蛍光顕微鏡を用いた。ろ過量を 1 mL または 50 mL とし、計数を観察倍率 1,000 倍で 15 視野（0.15 mm^2）とした。したがって検出限界は、サンプルＡ：2×10^2 cells/mL、サンプルＢ：2×10^4 cells/mL、サンプルＣ：2×10^2 cells/mL であった。マイクロコロニー法では SCD 培地上での静置条件を 30℃、6 時間とし、生じたマイクロコロニーを SYBR Gold で染色後、蛍光顕微鏡下で計数した。ろ過量を 10 倍希釈した試料を 1 mL または希釈しない試料 10 mL とし、計数を観察倍率 200 倍で 15 視野（3.75 mm^2）とした。したがって検出限界は、サンプルＡ：8×10^2 mCFU/mL、サンプルＢ：4×10^4 mCFU/mL、サンプルＣ：8×10^2 mCFU/mL であった。なお、一部の実験参加者では同時にマイクロコロニー自動計数装置を用いて計数を行った。平板培養法では SCD 培地での培養条件を 30℃、３～５日間とし、生じたコロニーを目視で計数した。参加者には菌数を伝えずに個々にブラインド試験を行った。データの解析にあたっては、蛍光顕微鏡操作に習熟した者の群としていない者の群に分けた。

Ｃ．研究結果

１．細菌迅速試験法技術研修会の参加者を表 1 に記した（公的研究機関：4 名、公益法人：3 名、民間企業：11 名、計 18 名)。

２．上記参加者に対して行った細菌迅速試験法に対するアンケートの結果を表 2 に示した。

３．細菌迅速試験法の共同実験の参加者を表 3 に、結果を表 4、図 1 および図 2 に示した。まとめを以下に記した：①蛍光顕微鏡操作に習熟している者の群では蛍光顕微鏡法での相対標準偏差（標準偏差／平均値）は 18%～31%（平均 26%）であったのに対し、習熟していない者の群では 33%～78%（平均 60%）であった。②平板培養法（混釈法）の相対標準偏差は、22%～41%（操作の習熟度を一定と見なし群に分けずに解析；平均 32%）であった。これは蛍光顕微鏡操作に習熟している者の群での蛍光顕微鏡値の相対標準偏差と同等であった。③蛍光顕微鏡操作に習熟している者であっても、蛍光顕微鏡 1 視野あたりの細菌数が約 80 の場合に、1 視野あたりの細菌数が約 30 の場合に比べて、計数値の相対標準偏差が小さくなった。④蛍光活性染色法に比べ、マイクロコロニー法の方が相対標準偏差が小さくなった。この傾向は蛍光顕微鏡の操作の習熟度にかかわらず見られた。⑤マイクロコロニーの計数にあたり、自動計数装置を用いた場合の相対標準偏差は、蛍光顕微鏡を用いた場合の相対標準偏差と同じ、または

小さくなった。ただし、今回の比較は蛍光顕微鏡操作に習熟している者のみの結果であるため、今後、習熟していない者についても、確認する必要がある。

D．考　察

1．細菌迅速試験法の技術研修会参加者へのアンケートの結果、①蛍光活性染色法とマイクロコロニー法のいずれの手法にも高い関心が持たれていること、②両手法の迅速性・簡便性が評価されていることが確認できた。また一般化にあたっては、蛍光顕微鏡操作の簡便化と計数基準の明確化が課題であることがわかった。菌数モニタリングにおいては、個々について計数値を詳細に求める方法とともに、菌数を－、±、＋、＋＋等の段階で評価し、菌数変化の傾向を求める方法も、迅速かつ簡便なモニタリングには役立つものと考えられる。

2．細菌迅速試験法の共同実験の結果、操作の習熟により、計数値の個人差は小さくなった。この理由として、操作に慣れることにより、①細菌と細菌以外の粒子の判別が明確にできるようになる、②焦点合わせの操作等が早くなることにより、蛍光の褪色を抑制できることが考えられた。また計数値の相対標準偏差を小さくするには、1 視野あたりの細胞数を 30 以上に調整すればよいと考えられた。

3．蛍光顕微鏡操作の習熟度にかかわらず、蛍光活性染色法に比べてマイクロコロニー法の計数値の相対標準偏差が小さくなった理由として、マイクロコロニー法では計数対象が 10～100 μm と大きく、また蛍光強度も強いために、検出対象を認識しやすいためであると考えられた。

E．結　論

蛍光活性染色法やマイクロコロニー法は、迅速かつ簡便な細菌試験法として、微生物学の基礎的な手法を習得した者であれば実施可能であることがわかった。また、自動計数装置の使用により計数操作の標準化が可能であること、蛍光顕微鏡を用いて計数する場合でも操作に習熟することにより計数値の個人差が軽減することがわかった。

F．参考論文、学会発表

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添付資料

表 1．細菌迅速試験法の技術研修会参加者．

所属	氏名
国立医薬品食品衛生研究所	棚元憲一
国立感染症研究所	佐々木 次雄
大阪府立公衆衛生研究所	皐月 由香
大阪府立公衆衛生研究所	山崎 勝弘
独立行政法人 医薬品医療機器総合機構	但野 恭一
財団法人 日本食品分析センター	関口 道子
財団法人 日本食品分析センター	山崎 健一
アステラス製薬株式会社	宮部 孝彦
エーザイ株式会社	鈴木 勝久
ＭＰテクノファーマ株式会社	山本 勝弘
ＭＰテクノファーマ株式会社	中山 雅章
協和発酵工業株式会社	栃木 公太
塩野義製薬株式会社	亀井 みゆき
武田薬品工業株式会社	岡 由子
田辺製薬株式会社	川上 公範
日本新薬株式会社	五島 隆志
扶桑薬品工業株式会社	西岡 吾郎
明治製菓株式会社	石渡 信由

表 2．細菌迅速試験法技術研修会参加者に対するアンケート結果（回答者 15 名）．

１）興味ある手法　蛍光活性染色法　８名
　　　　　　　　　マイクロコロニー法　８名

２）新手法に対するコメント
- 結果が迅速に得られるので，モニタリングに適している
- 操作が予想していたよりも簡単であった
- 培養法で検出できない細菌を検出できそうである
- 慣れれば有効に利用できそうである

３）今回の手法で難しかった点
- 蛍光顕微鏡での計数基準がわかりにくい
- 蛍光顕微鏡の操作（焦点合わせ等）がしにくい
- マイクロコロニー形成時間の基準値を決めてほしい
- 計数値に自信がもてない
- 試薬の染色性能試験法があれば良い
- 手法によって測定結果が変わるので，「真の値」がわからない

表３．細菌迅速試験法の共同実験参加者．

所属	氏名
国立感染研究所	栃木 公太(協和発酵工業株式会社)
財団法人 日本食品分析センター	山崎 健一
アステラス製薬株式会社	宮部 孝彦
エーザイ株式会社	鈴木 勝久
塩野義製薬株式会社	亀井 みゆき
株式会社日本食品エコロジー研究所	赤松 清
株式会社日本食品エコロジー研究所	藤原 由美
武田薬品工業株式会社	金井 芳則
田辺製薬株式会社	川上 公範
日東メディック株式会社	西田 真理子
日本新薬株式会社	五島 隆志
扶桑薬品工業株式会社	西岡 吾朗
ライオン株式会社	高橋 健治
ロート製薬株式会社	辻本 恵子
大阪大学大学院薬学研究科	一條 知昭
大阪大学大学院薬学研究科	王 暁丹
大阪大学大学院薬学研究科	見坂 武彦
大阪大学大学院薬学研究科	馬場 貴志
大阪大学大学院薬学研究科	山口 進康

表４．蛍光活性染色法、マイクロコロニー法、平板培養法により求めた細菌数測定結果における相対標準偏差（標準偏差／平均値）．

（１）蛍光顕微鏡操作に習熟していない者の群

サンプル	マイクロコロニー法	CFDA染色	DAPI染色
A	54	78	64
B	33	76	57
C	—	—	—

（２）蛍光顕微鏡操作に習熟している者の群

サンプル	マイクロコロニー法	CFDA染色	DAPI染色
A	24	—	31
B	18	33	25
C	—	—	—

（３）平板培養法（操作の習熟度を一定と見なし群に分けずに解析）

サンプル	平板培養法
A	41
B	22
C	—