



Original article

Experience of contamination during autologous cell manufacturing in cell processing facility under the Japanese Medical Practitioners Act and the Medical Care Act



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ABSTRACT

Cell therapy and regenerative medicine technologies require strict cell manufacturing procedures to be defined and addressed. Maintenance of the aseptic environment is critical to preclude extrinsic contamination risks, similar to conventional pharmaceutical manufacturing. However, intrinsic contamination risks exist in all cell manufacturing processes owing to the use of cells as the raw materials that cannot be sterilized, thus giving rise to the primary and secondary risks of cell contamination and cross-contamination, respectively. Analysis of contamination risks was conducted on experienced batches (29,858 batches) for the production of immune cells derived from autologous blood mononuclear cells under the Medical Practitioners Act and the Medical Care Act in Japan. From these batches, 0.06% (18 cases) of contamination occurred, representing low probability of contamination incidence during cell processing. Almost all the causes of these contaminations were regarded to be from the collected blood (intrinsic contamination), and subsequent cross-contaminations were prevented, considering that the secondary contamination risk can be reduced by adequate managements of operational procedures for changeover in aseptic environment.

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1. Introduction

A new regulatory framework for regenerative medicine including cell therapies has been implemented in Japan by two new laws that came into effect on November 25, 2014. There are known as the 'The Act on the Safety of Regenerative Medicine' in the Medical Practitioners Act, and the Medical Care Act, and the 'Pharmaceuticals, Medical Devices and Other Therapeutic Products Act' in the Pharmaceutical Affairs Act [1,2]. These laws are considered to expedite the industrialization of services for regenerative medicine [3], and many companies have already entered cell/tissue manufacturing fields. However, experience is limited for quality management in manufacturing living cell/tissue products. Consequently, many hurdles exist to overcome the

manufacturing of regenerative medicine products, compared with in conventional aseptic pharmaceutical manufacturing.

To produce cells in terms of safety as well as efficacy, cell manufacturing is a critical step to consider. Aseptic assurance is an important factor for manufacturing living cell/tissue products that cannot be sterilized. The quality system for aseptic manufacturing is planned around area design in the cell processing facility (CPF) for adequate maintenance of cleanness, and operational management to preclude extrinsic contamination risks in the process, similar to conventional pharmaceutical manufacturing. However, at initiation of human cell processing, the raw material has an intrinsic contamination risk from microorganisms originating from the donor or the environment during biopsy harvesting. This intrinsic contamination brings a primary risk in the cell manufacturing process, leading to secondary risk of cross-contamination to other products.

Cross-contamination is caused mainly in two ways; a splash mist of waste medium from the original culture vessel transferred directly through the air flow in a biosafety cabinet (BSC) into others during medium change operation, or the mist adheres onto the BSC

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floor or the operator's hands, being indirectly transferred into other vessels. Detection of cross-contamination is often delayed, leading to a broader expansion of contamination, which is a critical concern.

During autologous cell processing in Japan, the manufacturing process is currently carried out manually in a commercial BSC, and cultures of multiple patient cells are handled sequentially in a single BSC, which differs substantially from typical pharmaceutical manufacturing methods. The prevention of intrinsic contamination risks is one critical issue in autologous cell processing, and the prevention of cross-contamination during changeover, which requires cleaning after the completion of operation for each culture, is an important procedure for quality control [4].

Although many cell manufacturing cases exist for clinical treatment under the Medical Practitioners Act and Medical Care Act, experience of cell manufacturing under the Pharmaceutical Affairs Act is not extensive in Japan. Therefore shared knowledge in practical manufacturing is invaluable for the design of quality management. The Biotherapy Institute of Japan (BIJ) has manufactured approximately 30,000 batches of cells in the latest 5 years for immunotherapy within various clinical institutions. During the preparation of activated immune cells from peripheral blood at BIJ, the occurrence of contamination by proliferative microorganisms is observed visually, because no antibiotics are added to the culture medium during any stage of the process. Therefore, this study aimed to predict whether contamination risks during autologous cell manufacturing could be determined by confirming the contamination periods and sources observed during operation.

2. Materials and methods

2.1. Design of clean areas in the CPF

The floor plan of the CPF at BIJ, which has previously been used to manufacture cells, is shown (Fig. 1). Briefly, three cell processing

rooms exist, consisting of the aseptic area in a BSC (cleanliness ISO class 5) and a cell processing area (cleanliness ISO class 7), coupled with anterior/posterior buffer areas, isolated from the outside environment by a clean corridor with a changing room (cleanliness ISO class 8). Six BSCs were installed in each cell processing area. Thus 18 aseptic areas of BSCs were installed in the CPF for cell manufacturing.

2.2. Operational procedure in the cell processing room

In each cell processing room, one operator was engaged at a single BSC, with up to six operators entering one room at the same time. Each operator wore a whole-body lint-free suit, with a mask and gloves, and was then able to treat cells in the aseptic area. A centrifuge and four CO₂ incubators, with a BSC, were used by one operator as an individual operating area (Figs. 2 and 3). Operators were able to carry around culture vessels only in the range of the operating area to avoid any intersection of the operational flow lines. All culture vessels conformed to an airtight system, and open operations were carried out only in aseptic areas. After each operation, the operator cleaned the operating area using a certificated disinfecting procedure and sterilant to complete the changeover process in preparation for the next operation. Each operator handled a single batch during an operation and was not allowed to make parallel operations for multiple batches.

2.3. Expansion of activated immune cells

The procedure for the expansion of activated immune cells using an expansion kit (BINKIT[®], BIJ, Japan) is described below [5,6]. A description of the quality tests used to analyze surface markers is omitted in this report. Blood mononuclear cells were isolated from 50 mL of peripheral blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Sweden). A tiny quantity of the

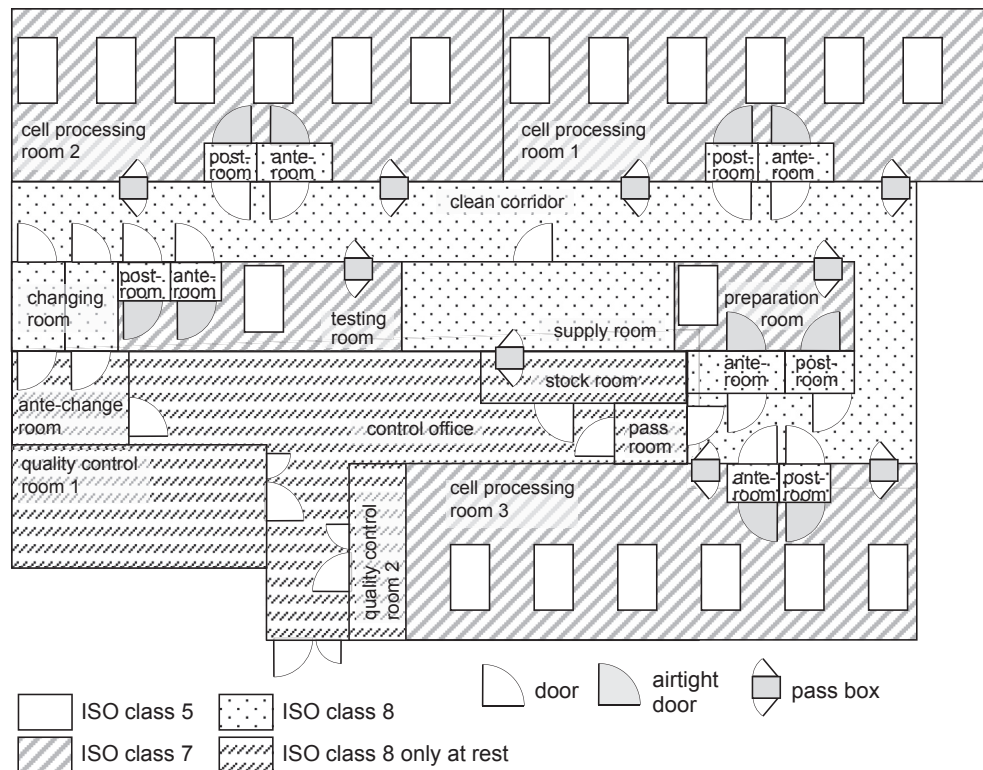


Fig. 1. Three cell processing rooms in the facility included the operating areas (cleanliness ISO class 7), coupled with anterior/posterior buffer rooms, isolated from the outside by a clean corridor with a changing room (cleanliness ISO class 8). There were a total of 18 aseptic areas (cleanliness ISO class 5).

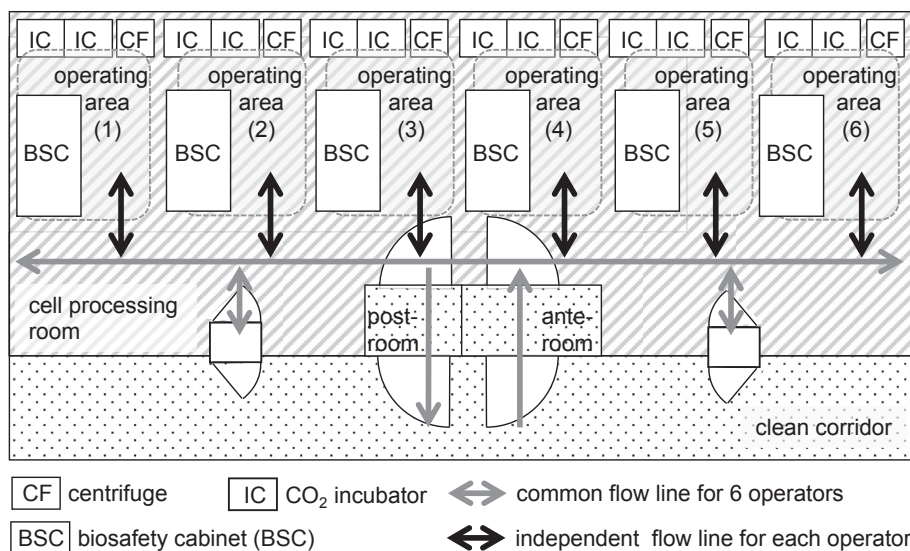


Fig. 2. Six BSCs, were used as the aseptic areas for operation in each cell processing room. A centrifuge, four CO₂ incubators, and a BSC were used by one operator as an individual operating area. The flow line for each individual operating area was isolated from the common flow line.

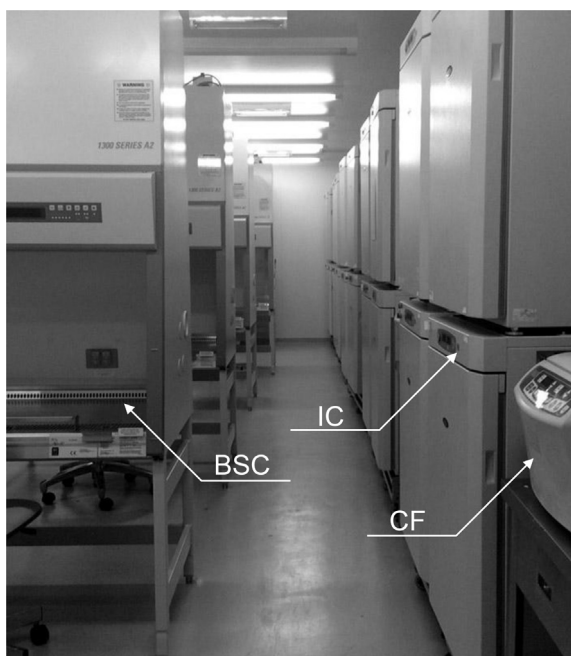


Fig. 3. Each flow line in the operating area was designed not to cross each other, and the other operators could not approach the area of the operator that had processed the cells at the BSC. The changeover process of each operating area was carried out after disinfection.

peripheral blood, which was collected from a patient, was checked the infection of fungi or bacteria by culture method at 30 °C using a sheep blood agar plate (Eiken Chemical, Japan). The isolated peripheral blood mononuclear cells were cultured in BINKIT[®] initial medium, in a BINKIT[®] culture flask for initial activation, with the addition of 5% heat-inactivated autologous plasma in a BINKIT[®] initial cocktail. Antibiotics, antibacterial or antifungal agents were not contained in the BINKIT[®] expansion kit. After 3 days of cultivation for activation, the cells were transferred to a culture flask containing BINKIT[®] subculture medium. Fresh culture medium was added to the flask every 2 or 3 days. When the number of cultured cells started to logarithmically increase, the cells were transferred

to a culture bag (NIPRO, Japan) until the end of culture. Incubation was performed at 37 °C in a humidified atmosphere of 5% CO₂ in air using an incubator (Astec, Japan). The total culture period was around 3 weeks, and activated immune cells proliferated to approximately 2×10^9 cells, with patient variability. After filling an infusion bag (Terumo, Japan) with Ringer's solution (Otsuka Pharmaceutical, Japan), the final product was shipped to the medical institution. For all final products, a sterility test, a mycoplasma negative test, and an endotoxin test were carried out. Briefly, an infection of fungi and bacteria was checked by plating method at 30 °C culture by using soybean-casein digest [SCD] medium (Eiken Chemical, Japan), which the culture period was up to 2 weeks. Mycoplasma detection was conducted by polymerase chain reaction [PCR] method using Cycleave[®] PCR Mycoplasma Detection Kit (Takara Bio, Japan). The sensitivity of endotoxin was checked by gel-clot method of limulus amoebocyte lysate [LAL] assay using Limulus ES-2 Test wako (Wako Pure Chemical, Japan). The positive sample would be rechecked for quantify by turbidimetric method when gelation was found.

2.4. Detection of fungi and bacteria contamination

The occurrence of contamination by proliferating fungi and bacteria during the process was detected by visual observation. At the start of each process, the operator confirmed the transparency, color and pH of the medium. If either the transparency or pH was changed from the reference, the turbid state and the culture day were recorded alongside confirmation. A sample solution of the medium was harvested to determine if contamination had occurred. If contamination was confirmed, the type of bacteria or fungal contaminated source was identified by direct microscopy of the microbiological culture.

2.5. Classification of contamination cases by manufacturing phase

The timing of contamination was classified into early, middle and late phases by culture days when a turbid state was recognized. The early phase was defined in the range of processing time up to 3 days for expansion, including the cell isolation and primary culture for activating the separated mononuclear cells. The middle phase was defined in the range from 3 to 7 days, including passage culture

up to the point of bag culture processing. The late phase was the remaining period until the end of the manufacturing process.

3. Results

3.1. Incidence of contamination in the manufacturing process

To prepare activated immune cells for clinical use 29,858 batches were manufactured in a CPF at BIJ during five years from 2010 to 2014 (Table 1). Cell processing of more than 15 batches was carried out daily in each BSC. Identification of fungi and bacteria for the final product was performed by sampling the culture medium before shipment, and check for mycoplasma infection and endotoxin assay were carried out simultaneously. Biological infection of all the final products, which the turbid samples were excluded, was denied and the sensitivity of endotoxin was negative at all points by gel-clot method of LAL assay in this study. To clarify biological contamination of fungi and bacteria in process, culture vessels for each batch were checked briefly and almost daily. The visual turbidity test was conducted when the color of culture medium was different from the usual. Eighteen cases (0.06%) were detected through the visual turbidity test, and contamination was confirmed in all turbid samples (Table 2). Contamination occurred in five cases during the early phase, and in 13 cases during the middle phase. There were no cases of contamination in the late phase. All contaminated products were detected before shipment by visual turbidity test as well as sterility test. Meanwhile, no fungi or bacteria were detected on checking of each peripheral blood.

3.2. Identification of proliferating fungi and bacteria in the contaminated cases

Identification of the source of contamination was carried out (Table 3). In only one case (No. 6), the fungus *Candida guilliermondii* was observed as the likely pathogenic. All other contaminations were caused by general bacteria, indigenous to humans. The most commonly found bacteria were the *Staphylococcus* genus confirmed in eight cases. *Staphylococcus* normally resides on the skin and mucous membranes of humans. *Citrobacter*, *Enterococcus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter* constitute part of the gut flora, whilst *Pseudomonas aeruginosa*, *Acinetobacter*, *E. coli*, and *Enterobacter* also exist in the natural environment, such as in soil or water.

4. Discussion

From the 29,858 cell-processing batches collected as a blood raw material in this study, the possibility of contamination was measured at 0.06%. The Red Cross Society of Japan has reported that the past bacterial positive rate for blood transfusion preparations, containing the first collected blood, was also 0.06% [7]. This is considered to be low because of the quality management employed according to the good manufacturing practice (GMP) under the pharmaceutical law. Of note, Tanokuchi et al. reported

Table 2
Contamination incidence observed in the manufacturing process.

Phase of the process	Culture days	Year					Total
		2010	2011	2012	2013	2014	
Early phase	1–3	0	2	0	3	0	5
Middle phase	4–10	3	3	5	0	2	13
Late phase	11–	0	0	0	0	0	0
	Subtotal	3	5	5	3	2	18

the possibility of bacterial contamination at a university hospital in Japan as being 0.18% positive [8]. In the present study, additional risks of contamination arose not only from the cell source harvested from patients but also from the long-term processing for cell expansion, suggesting that a contamination risk of 0.06% is low enough to make a clinical application. Interestingly, it cannot be denied that the mononuclear cells processed in this study reduced the possibility of contamination because of the presence of white cells in the blood that are reported to impede bacterial contamination [9,10].

The phase of timing was classified by the risk analysis (Table 4). The early phase is a primary period, which has extrinsic risk operations at the cell isolation and the passage in culture. Murphy et al. reported that contamination of collected blood occurred after 4 days storage [11], that an interfusion at blood collection could not be detected at the early phase. The middle phase is a period for stabilization, which has operations for passage to change vessels from a flask to a culture bag. The possible cause is both intrinsic and extrinsic contamination. The late phase is the period for proliferative process in a culture bag after confirmation of logarithmically increase of cells. The possibility of contamination is low since all the operations in this period are to add culture medium using a semi-closed tubing circuit, whereas the risk is extrinsic only.

In the early phase, five cases of contamination were observed, and all of which were detected as common bacteria found in the human intestines. This suggests that bacteria that reside on the skin of the patient would not be detected by visual observation owing to the low sensitivity against small quantities of bacteria such as skin flora, which originated from the venipuncture plug [12]. These confirmed intestinal bacteria likely existed in the blood of the patients prior to blood collection, resulting from the occurrence of sepsis caused by medical/cancer treatments. To prevent the contamination caused by microorganisms existing in blood, it is necessary to add bacteriostatic activity at the cell expansion procedure in correspondence to high-risk patients.

During the middle phase, two major contamination risks procedures are considered as shown in Table 4. One is the intrinsic contamination in raw materials contaminated by an existence in blood or an interfusion from the patients' skin during harvesting; the other is extrinsic risks from the external environment, including the operators during cell processing. The turbid state caused by the venipuncture plug is considered to occur after 4 days or later, moreover the incident in this study has a low probability. In addition, *C. guilliermondii* (case No. 6) and *Klebsiella pneumoniae* (case No. 8) shown in Table 3 are likely intrinsic contaminants in the blood because No. 6 was likely pathogenic and No. 8 is an intestinal bacterium observed in the initial early phase. Therefore the majority of contaminated sources, which is detected at the middle phase, are intrinsic in the raw materials. The *Staphylococcus* genus found in eight cases is considered to be intrinsic owing to interfusion at the point of blood collection, and the other confirmed bacteria were also considered intrinsic either from the blood or the interfusion, because no occurrences of

Table 1
Activated immune cell manufacturing performance in BIJ.

Activated immune cells	Year					Total
	2010	2011	2012	2013	2014	
Natural killer (NK)	3722	3017	3427	3660	3059	16,885
Others	3339	2241	2612	2710	2071	12,973
Subtotal	7061	5258	6039	6370	5130	29,858

Table 3
Identification of microorganisms in the observed contamination cases.

No.	Times of the manufacturing		Days of turbid state observed	Phase of the process	Confirmed microorganisms	Classification of microorganisms
1	2010	Mar	7 days	Middle phase	<i>Staphylococcus sp.</i>	Gram-positive bacteria
2		Apr	9 days	Middle phase	<i>Staphylococcus sp.</i>	Gram-positive bacteria
3		May	8 days	Middle phase	<i>Staphylococcus sp.</i>	Gram-positive bacteria
4	2011	Apr	7 days	Middle phase	<i>Pseudomonas aeruginosa</i>	Gram-negative bacteria
5		Jul	3 days	Early phase	<i>Citrobacter freundii</i> & <i>Acinetobacter sp.</i>	Both gram-negative bacteria
6		Oct	7 days	Middle phase	<i>Candida guilliermondii</i>	Fungi
7		Nov	2 days	Early phase	<i>Enterococcus</i>	Gram-positive bacteria
8		Dec	5 days	Middle phase	<i>Klebsiella pneumoniae</i>	Gram-negative bacteria
9	2012	Jan	8 days	Middle phase	<i>Acinetobacter sp.</i>	Gram-negative bacteria
10		Mar	6 days	Middle phase	<i>Staphylococcus epidermidis</i>	Gram-positive bacteria
11		Apr	6 days	Middle phase	Coagulase-negative Staphylococci	Gram-positive bacteria
12		Sep	8 days	Middle phase	<i>Staphylococcus epidermidis</i>	Gram-positive bacteria
13		Nov	7 days	Middle phase	<i>Staphylococcus epidermidis</i>	Gram-positive bacteria
14	2013	May	2 days	Early phase	<i>Klebsiella pneumoniae</i>	Gram-negative bacteria
15		Oct	1 day	Early phase	<i>Escherichia coli</i>	Gram-negative bacteria
16		Dec	1 day	Early phase	<i>Escherichia coli</i>	Gram-negative bacteria
17	2014	Apr	8 days	Middle phase	<i>Enterobacter cloacae</i>	Gram-negative bacteria
18		Jun	7 days	Middle phase	<i>Staphylococcus epidermidis</i>	Gram-positive bacteria

Table 4
Risk analysis of the activated immune cell manufacturing process.

Classification of the process phase	Days of culture	Operation of the process	Culture vessel / First-order container	Contamination risk detectable in each phase
Early phase	(0)	(Acceptance of peripheral blood) - isolation of mononuclear cells - primary culture (Activation of mononuclear cells)	blood collection tubes ↓ a flask for activation	Intrinsic with raw materials - existing in blood
Middle phase	3 4~	- passage culture - adding of medium (Logarithmically increase of cells) - final passage for cell expansion culture	culture flasks ↓ a culture bag	Intrinsic with raw materials - existing in blood - interfusion at blood collection
Late phase	10 11~	- adding of medium	↓ an infusion bag	Extrinsic from environment
Shipping	(~21)	- washing/packaging (Quality test)	↓	Extrinsic from environment

contamination were confirmed in the late phase. Meanwhile, it cannot be denied completely that the contamination occurred in the isolation process of mononuclear cells because the isolation procedure is the most high-risk operation in the processes of cell expansion. The results of the late phase suggest that the risk of extrinsic contaminated sources in the manufacturing process is low.

In this study, all contaminations were observed in early and middle phases, suggesting likely intrinsic contamination risk following medical treatment prior to blood collection. Furthermore, the extrinsic contamination risks were considered to be controlled sufficiently with proper aseptic processing and changeover methods under the certificated procedures. Thus, all the contaminations were caused by primary contamination following medical treatments prior to blood collection, and the risk of cross-contamination by secondary contamination could be prevented. This suggests that airtight culture vessels including culture flasks and bags, which have openings to make small and limited apertures at the aseptic area, can help to reduce both secondary and the extrinsic contamination risks effectively. However,

this doesn't imply complete elimination of extrinsic contamination, because the confirmed fungi and bacteria were able to proliferate at 37 °C in the cell culture medium with the blood mononuclear cells. Consequently, the sterility test of the final products should be performed by low volume sampling, based on quality management.

5. Conclusions

In this study, incidences of contaminated sources were evaluated to identify contamination risks during the manufacturing process of activated immune cells, performed in one company over 5 years. The incidence of contamination (0.06%) was relatively low, and the occurrence of cross-contamination (secondary contamination risks) could be eliminated. This suggested that most of the contaminated sources were due to intrinsic microorganisms in the donor patient raw materials. The secondary or extrinsic contamination risks could be reduced by adequate management of the aseptic environment and operational procedures including aseptic processing and changeover.

Conflict of interest

The authors have no conflict of interest to declare.

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