Temperature and holding time of instrument sterilization as an infection control of odontectomy

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ABSTRACT

Odontectomy should be performed aseptically. The goal of sterilization is the complete killing of all forms of microbial life including bacterial spores on the items being processed. Biologic monitoring provides the main quarantee of sterilization. The aim of this study was to find the interrelation of the temperature and the holding time of instrument sterilization as an infection control for the successful of lower molars odontectomy. This experimental laboratory study was conducted at the Oral Maxillofacial Surgery Department in the Hasan Sadikin General Hospital Bandung and at the Microbiology Laboratory Faculty of Dentistry, Universitas Padjadjaran, Jatinangor. The Protocol was performed in three methods of sterilization: dry heat with oven and ozon, dry heat with oven and infrared (125°C for 15 minutes), both were monitored by *Bacillus atrophaeus* as the biologic indicators, and autoclavization (121°C for 15 minutes) with *Geobacillus stearothermophilus* as the biological monitoring, with 17 times repetition. After sterilization, all of the indicators were cultured on Nutrient Agar Plate (NAPs), and the subsequent growth was assessed. The colony forwing units (CFUs) were counted by Stuart Electric Bacteria Colony Counter. Adequate positive and negative controls were used in every cycle. The results showed that after autoclavization, all spores were killed. In comparison with dry heat in oven, there were still CFUs on the NAPs, but no colonies grow after 3 repetitions by oven and infrared. Heating in oven and ozon could only reduce the spore numbers, even after repeating 5 times. The reduction of the CFUs were greater in more repetition. According to the statistical analysis, the differences were significant. This study concluded that sterilization by oven and infrared will be achieved after 3 holding times (30-35 minutes) and dry heat with oven and ozon could only act as germicide. In autoclavization all of *Geobacillus stearothermophilus* have been killed.

Key words: *Bacillus atrophaeus, Geobacillus stearothermophilus*, dry heat sterilization, autoclavization, time and temperature

ABSTRAK

Odontektomi harus dilakukan secara aseptik. Tujuan sterilisasi adalah untuk membunuh seluruh mikrob hidup termasuk spora bakteri. Monitor biologis memberikan jaminan utama untuk sterilisasi. Tujuan penelitian ini adalah untuk menemukan saling hubungan antara suhu dan lamanya waktu sterilisasi alat sebagai kontrol infeksi untuk keberhasilan odontektomi gigi molar bawah. Penelitian laboratories

**Correspondence author: Florence Meliawaty, Department of Oral and Maxillofacial Surgery Faculty of Dentistry Universitas Padjadjaran Jl. Sekeloa Selatan No. 1 Bandung, West Java-Indonesia, Tel./Fax: +6222-2504985/2532805*

eksperimental ini dilakukan di Bagian Bedah Mulut dan Maksilofasial Rumah Sakit Umum Dr. Hasan Sadikin dan di Laboratorium Mikrobiologi Fakultas kedokteran Gigi Universitas Padjadjaran, Jatinangor. Cara penelitian dilakukan dengan tiga metode sterilisasi: pemanasan kering dengan oven dan ozon, pemanasan kering dengan oven dan infrared (125° C selama 15 menit), keduanya dimonitor oleh Bacillus atrophaeus sebagai monitor biologis, dan pemanasan dengan autoklaf (121° C selama 15 menit) dengan Geobacillus stearothermophilus sebagai monitor biologis, dengan 17 kali pengulangan. Setelah sterilisasi, semua indikator dikultur pada Nutrient Agar Plate (NAPs), dan urutan pertumbuhannya diperiksa. Colony forwing units (CFUs) dihitung dengan Stuart Electric Bacteria Colony Counter. Kontrol positif dan negatif yang memadai digunakan pada setiap siklus. Hasil penelitian menunjukkan bahwa setelah pemanasan dengan autoklaf, semua spora mati. Dibandingkan dengan pemanasan kering dengan oven, masih terdapat CFUs dalam NAPs, tetapi tidak ada koloni bakteri yang tumbuh setelah 3 kali pengulangan dengan oven dan infrared. Pemanasan dengan oven dan ozon hanya dapat mengurangi jumlah spora setelah lima kali pengulangan. Pengurangan CFU makin banyak setelah beberapa kali pengulangan. Berdasarkan analisis statistik, terdapat perbedaan yang bermakna. Penelitian ini menyimpulkan bahwa sterilisasi dengan oven dan infrared dapat tercapai setelah tiga kali pengulangan (30-35 menit) dan pemanasan kering dengan oven dan ozon hanya dapat berperan sebagai germisid. Pemanasan dengan aoutoklaf menyebabkan semua Geobacillus stearothermophilus mati.

***Kata kunci**: Bacillus atrophaeus, Geobacillus stearothermophilus, sterilisasi pemanasan kering, autoklaf, suhu dan lama waktu*

INTRODUCTION

Dentists have high risk to be infected by a disease, especially diseases that transfected by saliva, and blood, such as Hepatitis B, and HIV.[1,2] The HIV virus spread from patient to patient in Surgery Department of New South Wales, Australia. in November 1989 was reported five from nine patients (55.56%) in the same day of their treatment became HIV positive after removal of cysts and minor surgeries, even though the operators were still HIV negative.[1]

In Oral and Maxillofacial Surgery Department, lower third molar removal is the most frequent procedure. Succeeded operation had to be achieved by proper procedures and techniques, adequate operator skill, also aseptic technique as a preventive measure of post operation infection.[1] In oral surgery procedures, tools will be in contact with oral tissues/opened oral mucosa, so it has to be sterilized tools and materials.[1] The complication prevalence of lower third molar removal in form of infection in Brazil as much as 4.6-30.9%.[3,4]

Preliminary survey conducted in Oral and Maxillofacial Surgery Department of Dr. Hasan Sadikin General Hospital (RSHS) Bandung related to the effort of detecting the possibility of post odontectomy procedures infection in period of September 2009-January 2010 there were 17 out of 55 odontectomy cases (30.91%) had the post operation infection, even though the patient had underwent 5 days regime of antibiotics.

Patient oral cavity is a home for complexes of microorganisms and are able to cause infection after an oral surgery procedure. Though patient oral cavity generally is the main source of pathogenic microorganism in dentistry, it is not possible to precisely detect what microorganism the patient carries. Often the patient did not realized that they had the disease, or they were too embarrased to admit that. Therefore, it is important to prevent the pathogenic microorganism spread in dentistry. It is a must to apply the infection control procedures based on the universal precaution measure, which applied generally for every patient, by pretending that every patient was infected by pathogenic microorganism. At least, this procedure shall be applied as a standard of infection control for every patient in oral and dental treatment, so it called as standard precaution.

This fundamental will be very wise and do not require any more prevention measures, because it will protect every patient, dentist, and dental team, including dental assistant, dental laboratory technician and dental radiology. Be-

sides it will reduce the clinical staff worry and also prevent discrimination of treatment of patients.[1]

Based on this, the disease spread prevention in Oral and Maxillofacial Surgery Department is an absolute procedure to protect patient and dental team. The cross infection control success needs teamwork of every personnel in dental team at their procedures.

Sterilization process that commonly used is the wet heat which is the autoclave and the dry heat by oven. The oven commonly used has no thermometer, thus physically the sterilization state is hard to determine. The oven has 2 compartment, the upper part contains ozone while the lower part equipped with infrared. Wraps only used in autoclave sterilization because in the oven after sterilization the tools can be used directly. Autoclave is a costly sterilization device, while the oven equipped with ozone and infrared has already used in common as a sterilization device with lower price, but the steril level guarantee has not yet been studied. The autoclave heating is using temperature 121°C within 15 minutes, and for oven equipped with ozone and infrared using 125°C for 15 minutes.[5]

Theoretically, the sterilization through heating with oven can be done in 160°C for 2 hours or 180°C for 1 hour.[1,2] The temperature and time of sterilization differences are interesting to be discussed further. Continues use of device, will damaged it. Also, with the routine used sterilizator can be damaged/broken, such as leaks in sterilization so the temperature could not be reached, the contact time between the heat and tools was not adequate. As a result it will fail the sterilization process, thus the tools can not be used to patients.[4] In Norway, it was reported a failure of sterilization of 3 out of 163 autoclaves (1.8%) and 14 out of 109 ovens (12.8%).[6]

Each tools sterility, can be tested microbiologically, but this way the tools can not be used to treat patients again. Therefore, it is need an indicator which can be used as a guarantee that the sterilization worked well. Sterilization process monitoring shall be based on 3 things, which are: physically by thermal measurement, pressure, and time[1]; chemically using the autoclave tape, sterilization pouch which showed color changes if the sterilization had completed; biologically using the spore strip or spore culture suspension; for autoclave it uses the *Geobacillus stearothermophilus*, while in the oven sterilization uses the *Bacillus atrophaeus*.[1,2,6] In the Oral and Maxillofacial Surgery Department of RSHS, only autoclave tape, and sterilization pouch are being used. Biological indicator is an absolute indicator to prove that the autoclave or oven sterilization monitoring, both worked well.

METHODS

As an examination material, it was used the post operation tools after odontectomy of lower third molar in Oral and Maxillofacial Surgery Department of RSHS Bandung. Tools and materials used in the clinic such as mouth glass, tweezers, explorer, scalpel/blade, bein, forceps, curette, needle, string, and syringe.

The sterilization device was the autoclave (Melag type MELAtronic® 23) as wet heating device and oven (Corona® ZTP 80A-7) as dry heat sterilizator equipped with ozone at the upper part and infrared at the lower part. Also tools and materials used commonly in microbiology laboratory such as test tube, petri dish, oven (Melag Sterilizer 255), microscope, electrical bacteria colony counter (dr. Stuart), incubator, object glass, filter paper, methylated lamp, sterile tweezers. Materials for Gram bacterial coloring were Carbolic Gentian Violet (CGV), lugol, alcohol 95%, fuchsin solution. For Klein bacterial coloring were carbolic fuchsin, $H_2SO_4$1% or alcohol 95% and blue methylene.

The bacterial culture mediums are Blood Agar Plate (BAP), Agar Plate (AP), bulyon. Biological indicator of this sterilization monitoring used are *Bacillus atrophaeus* (ATCC 9372), and *Geobacillus stearothermophilus* (Attest™ 3M 1262P, ATCC 7953).

Sample obtained from bulyon washed of 10 types of tools used on patients with odontectomy of lower third molar treatment in Oral and Maxillofacial Surgery Department of RSHS Bandung during November 2009-March 2010. The bulyon (0.1 ml) invested on the sterile BAP (blood agar) for bacterial colony counting of the washed materials. The rest of the washed materials were put into Erlenmeyer tube to be sterilized in oven with ozone and oven with infrared. Then, 0.1 ml bulyon was invested on the another sterile BAP.

This treatment was applied toward 17 patients of odontectomy.

In this preliminary study, all of the bulyon that had been sterilized did not showed any bacterial colony growth in every BAP culture, which means that the bacterias died after sterilization. Based on the theory, the most resistance bacteria toward sterilization is the spore type.[7]

Based on it, the sterilization was done toward the spore of *Geobacillus stearothermophilus* in autoclave and *Bacillus atrophaeus* in oven with ozone and oven with infrared in Oral and Maxillofacial Surgery Department of RSHS. If the spore was dead, any bacterias should dead. Bacteriological measurement was the colony counting (CC) of contaminant bacteria and was done in the Microbiology laboratory of UNPAD Dentistry in Jatinangor which had been preceeded with preliminary study since November 2009. Because of biological indicators being used and to be countable, the amount of the indicator was adjusted to the amount of contaminant bacterias which stuck with the tools: mouth glass, probe/explorer, tweezers, scalpel/blade, syringe, forceps, bein, curette, needle, and string.

Each tools were washed using the 10 ml sterilized bulyon in a large sterile petri dish. The washed water as an examination material (EM) was made suspension of homogenic bacteria. Examination material was taken 0.1 ml with disposable syringe then invested on the BAP evenly using the oese based on the lining method. The BAP culture was transported into the Microbiology Laboratory to be incubated in 37°C temperature for 18-24 hours. The next day, the colony growth on the BAP were counted. Colony counting (CC_1) of grown bacterial colony on the BAP used as the initial test bacteria which was *Bacillus atrophaeus* that will be sterilized using the oven with ozone, oven with infrared, and *Geobacillus stearothermophilus* for autoclave sterilization.

The biological indicator *Geobacillus stearothermophilus* was easily obtained in form of a self-contained vial (Attest™ 3M 1262P, ATCC 7953), while for the *Bacillus atrophaeus* (ATCC 9372) available in form of culture. Related with the sterilization of dry heat inside the oven then for sterilization monitoring, we used the dry spore strips. For this purpose the thick filter paper was cut into 1 x 1 cm size, and each of the filter paper put into the test tube, sealed then sterilized using the oven with 160°C for 2 hours.

Bacillus atrophaeus was suspended into bulyon equalized with the McFarland turbidity 0.5 and pipetted 0.1 ml, then it was invested evenly on the AP, incubated for 37°C for 18-24 hours. As a negative control, AP which not bacteria invested was also being incubated. By the next day the colony counting (CC_2) was conducted. Subsequently, another suspension of *Bacillus atrophaeus* was made with McFarland turbidity 0.5 and diluted serially. So, it would be even with the CC_1. Each of the suspension were pipetted 0.5 ml and put into the test tube with thick filter paper 1 x 1 cm size that had been sterilized (tube $A_1, A_2, \ldots, A_{17}$) and left to dry the spore strip. To obtain the microscopic figure of spore colored after sterilization, it was also made *Bacillus atrophaeus* suspension with turbidity Mc Farland 1, then mixed with the carbolic fuchsin solution evenly so there would be turbidity of McFarland 0.5. From this compound, it was pipetted 0.5 ml and absorbed into a sterile filter paper inside a test tube (tube $B_1, B_2, \ldots, B_{17}$).

Biological indicator for the autoclave was the spore strip of *Geobacillus stearothermophilus* in available pack (Attest™ 3M 1262P, ATCC 7953). Subsequently, the procedures would be the same with 1st method. The autoclave sterilization used temperature of 121°C for 15 minutes toward 17 packs of IB *Geobacillus stearothermophilus* available in market, as a repetition of treatment. For 2nd method, dry heat sterilization (oven and ozone) in 125°C for 15 minutes for tube A and B filled with spore strip of *Bacillus atrophaeus*. 3rd method: dry heat sterilization (oven and infrared) in 125°C for 15 minutes toward tube A and B filled spore strip *Bacillus atrophaeus*.

As a positive control, used one tube of A and B that was not sterilized, while for the negative control used the sterile filter paper drabbled with sterile bulyon in similar treatment. After sterilization, each of the A tube with spore strip including the positive and negative control were put into 5 ml sterile bulyon, then mixed using the vortex mixer so that the spore which stuck on the filter paper released and spreaded evenly inside the bulyon. Microsopically, it was done the Gram coloration and spore coloration according to Klein coloration.

Bacillus atrophaeus suspension in this tube was pipetted 0.1 ml, and invested in sterile AP, and incubated in 37°C for 18-24 hours. As a negative control for the culture medium used the AP from bulyon which not invested and incubated in the similar way. The next day, it was done the colony counting for each AP culture.

Assessment for the tube B after sterilized, it was added 1 ml of physiological NaCl, then it was prepared for the assessment using the Klein coloration technique, but the heating was done as the sterilization process using the oven 125°C for 15 minutes. This treatment repeated for 17 times for A and B tubes based on the sterilization 1st, 2nd, and 3rd method.

After autoclave sterilization, IB vial which contained *Geobacillus stearothermophilus* mixed the medium by pressing the vial/medium cap, so the inside part of the ampoule was broken. After that, the 0.1 ml of the vial invested on the AP based on the lining method and incubated in 55°C for one week. As a positive control, it also invested vial which not sterilized, while for the negative control used the AP which not cultured, but was incubated in the similar way. After incubation, it was done the colony counting with Electric Bacteria Colony Counter (dr. Stuart).

Because of sterilization with 2nd method (oven and ozone) and method 3 (oven and infrared) still had the bacterial growth indicator of AP culture, then the sterilization process with the oven continued. After the sterilization process finished, the "on" button of the oven became "off". To repeat the sterilization process, the "on" button was pressed again. Thus, it would be twice sterilization processes and by the similar way continued up to 3x, 4x and 5x, up to sterile condition achieved.

Based on the bacteriology assessment, after the Gram coloration, there was no bacteria and in the culture of AP had no colony growth. Colony counting was done by placing the culture of AP on the counting table. On this table, there were small squares for ease of counting process. To prevent double counting, the colony on the vertical lines would be counted as the left square colony, while the colony on the horizontal lines counted as the upper square. The difference of CC from every AP was counted and analyzed statistically.

RESULT

Gram coloration of biological indicator *Geobacillus stearothermophilus* which was not sterilized/had not yet sterilized and also as a positive control showed rod type bacteria with purple color, so that was Gram positive. In form of vegetative, there was an area of non colored with oval shaped. The spore coloration based on the Klein technique resulted red colored spore, located subterminally in form of vegetative, and more spores which were not rod type, became free spores.

Similar figure was also resulted from the *Bacillus atrophaeus* culture, but compared to the *Geobacillus stearothermophilus* with Gram coloration, *Bacillus atrophaeus* showed as rod type bacteria and arranged in chain formation, so that was called *streptobacillus*. In the AP, the *Bacillus atrophaeus* showed irregular border colony, with diameter of 2-5 mm. Also in the Klein coloration, the spore of *Bacillus atrophaeus* spore which subterminally located to the edge compared to the spore of *Bacillus stearothermophilus* which relatively centered.

Biological indicator culture that was not sterilized which was the positive control in AP showed colony growth around the spore strip. Besides of that, the spore suspension was created in the bulyon and was invested in the AP and resulted in colony spreaded all over the culture surface.

After treatment through autoclave sterilization process, biological indicator of *Geobacillus stearothermophilus* in its pack had

Table 1. Amount of *Bacillus atrophaeus* colonies before and after oven and ozone sterilization (n = 17)

Sterilization	Statistics measurement			
	Average	SD	Med	Mean
Before	312.6	44.0	301	237-389
After 1 times	292.2	41.6	283	220-361
After 2 times	269.2	42.4	261	201-333
After 3 times	248.4	41.5	240	187-312
After 4 times	229.6	41.2	223	170-295
After 5 times	206.5	37.0	203	152-272

Note: ANOVA repeated measures : F=15.54; p<0.001
Comparison between measurement based on the paired t test p<0.001

Table 2. Colony counting of *Bacillus atrophaeus* before and after sterilization with oven and infrared (n=17)

Sterilization	Statistics measurement			
	Average	SD	Med	Mean
Before	273.8	54.8	276	164-380
After 1 times	134.6	64.7	125	59-280
After 2 times	6.7	6.1	4	2-21
After 3 times	0	0	0	-

Table 3. Decreased colony counting of *Bacillus atropheus* colonies after oven sterilization

Sterilization	Decreasing of *Bacillus atrophaeus* (%)		Value P*)
	Oven + Ozon	Over + Infrared	
After 1 times	6.5 (2.7)	52.0 (16.1)	<0.001
After 2 times	14.0 (4.0)	97.7 (1.7)	<0.001
After 3 times	20.7 (5.4)	0	<0.001

Note: *) counted based on the unpaired t test

no color change, which means the sterilization process had worked well.[1] Gram coloration or Klein coloration for the biological indicator of *Geobacillus stearothermophilus* post autoclave treatment showed no bacteria or spore anymore. Also with the Gram coloration that was made of *Bacillus atrophaeus* spore strip after sterilization using the oven had no bacterial vegetative condition. On the Klein coloration, it was shown that the spore colored red without the vegetative form, unless after the oven and infrared sterilization process which was done 3, 4, and 5 times had no vegetative form not either bacterial spore on every examined preparate.

Invested examination materials on the BAP consisted of several different colonies. Generally the BAP cultures dominated with the small rounded colonies (pin-pointed), flat border, with 0.3-0.5 mm diameter, and green colored surroundings on the BAP cultures so there would be claimed as haemodigestive reaction. Another colonies were also rounded formation and had a relatively larger diameter (0.8-1 mm), without reaction toward the BAP, thus no haemolysis occurred. The colonies from Gram positive rods were rounded form but irregular border with diameter of 1.2-2 mm. Some of it showed haemolysis reaction, because the surroundings were became clear area.

While autoclave process was about to begin the thermometer showed 27°C, similar with the room temperature. After 15 minutes of heating, the autoclave temperature turned to 121°C and was stable up to 15-20 minutes on that temperature. The temperature decreased on 40th minute, while the drying process began at the 50th minute.

On the oven and ozone or oven and infrared heating, since the "on" button was pressed, the first sterilization took 15 minutes, then the "on" became "off". Then the "on" button could not be pressed up to 5 minutes, then the second sterilization process initiated but was only 5 minutes, and the "on" button turned to "off" again. Then we awaited for another 5 minutes, so that the third sterilization process could be done and was also took 5 minutes. This processes were repeatedly until the 5th sterilization process.

After autoclave process of the AP culture did not showing any colonies development of *Geobacillus stearothermophilus*, even though on the positive control of *Geobacillus stearothermophilus* was grown to be rough colonies, irregular formed, with diameter of 1-3 mm. On the negative control on the BAP which was also not cultured there were no colonies grown.

Before sterilization with the oven and ozone, the range of *Bacillus atrophaeus* colonies were 237-389 (Tab. 1). After first time sterilization the *Bacillus atrophaeus* colonies amount were decreased to 220-361 and more after the second sterilization (201-333). After the 3rd sterilization, it was even more decreased (187-312). The colony counting amount were getting smaller (170-295) after the 4th sterilization, and the most least was after the 5th sterilization (152-272). Also after the first sterilization, colonies were grown more closer each and the diameter were smaller, while after the 2nd and 3rd processes the colonies were larger.

Bacillus atrophaeus colonies which grown on the AP before sterilization with oven and infrared and also as a positive control, had ranged 164-380 (Tab. 2). After the 1st sterilization the amount was reduced to 59-280. After the second sterilization it was only 2-21, after 3rd sterilization there were no colonies of *Bacillus atrophaeus* grown on the AP culture. Also, after the 4th and 5th sterilization on every sterilized culture, had no grown bacteria colonies.

Bacillus atrophaeus colonies that grown on the AP after sterilized with oven and ozone were

smaller than before the sterilization process. Also with the colony counting after 2nd, 3rd, 4th, and 5th sterilization processes were smaller compared to the previous sterilization processes (Tab. 3). Even though it was not much different, but it was clear that after the 1st sterilization in oven and ozone there were decreased colony counting of 6.5%; larger after the 2nd sterilization (14%), and even more after the 3rd sterilization (20.7%). Otherwise, the decreased of colony counting amount resulted from oven and infrared in first sterilization, had the bigger value (52%), and this value reached its peak at 2nd oven and infrared sterilization (97.7%), because after the 3rd sterilization there were no spores grown on the AP.

DISCUSSION

Gram coloration made directly from the examination materials had shown the existence of *Streptococcus*. This result supported with colonies on Blood Agar which shown the haemodigestive surroundings around the colonies. *a streptococcus* culture resulted haemodigestion on the Blood Agar because this type of bacteria capable of turning haemoglobin (Hb) inside the blood cells to become met-haemoglobin, so it would resulted in green colored area on solid culture containing blood. This bacteria including the *viridans* group of *streptococcus* which were dominant inside the oral cavity. The amount of the *Streptococcus* relatively more compared to *Staphylococcus* or Gram positive rods. In the *viridans* group of *Streptococcus* it was including the *S. salivarius*, *S. sanguis*, *S. mitis*, and *S. mutans*.[8]

Biological indicator investment which had not been sterilized as a positive control of AP showed vegetative growth, either for the *Geobacillus stearothermophilus* or *Bacillus atrophaeus*. This concluded that the biological indicator using the spores which was capable of germination to return to its vegetative form. So, both of the biological indicators were reliable to be used in monitoring the result of sterilization processes. Otherwise, as a negative control which was the AP which had not invested any biological indicators after incubation had no grown colony, which meaned that the AP used was sterile and did not contained any other bacteria. By the result, it can be concluded that the AP was qualified as a medium which could be used in this study, because the bacteria was able to grow well on the AP medium.

After autoclave process, on the AP culture the *Geobacillus stearothermophilus* colonies were not grown. This condition concluded that the autoclave process that was done in 121°C temperature for 15 minutes resulted a sterile condition. If the spore bacteria could be dead, then another bacteria which had relatively lesser resistance should also be dead.

Otherwise, on the oven heating temperature of 125°C for 15 minutes, either oven and ozone, or oven and infrared could not be stated as sterile, because the AP culture still had *Bacillus atrophaeus* colonies growth, although on the Gram and Klein colorations there were no its vegetative form. This condition stated that heating with oven and ozone or oven and infrared with temperature of 125°C for 15 minutes had only killed bacteria in form of vegetative only, and could not destroy its spore. The spore could inititated another germination process if it was cultured on a new medium and incubated in prosperous environment. This result would also proved that the *Bacillus atrophaeus* spore could be used again as another monitoring for succesful control of sterilization process of the oven used.

On the Klein coloration, generally the spore bacteria suspension was mixed with the fuchsin carbolic in similar amount. Then, it was heated on water bath for 15-20 minutes. In this study, it was spored *Bacillus atrophaeus* which used, then it was absorbed to a sterile filter paper so it had no other contaminant bacteria. The sterilization process through oven heating with 125°C for 15 minutes would kill the bacteria, but still not able to kill the spore. Spore wall relatively had more thickness and contained calcium dipicolinate in large amount which inhibited the spore mortality process.[23] That is, why in the Klein coloration which was done right after the sterilization process on the spore strip, then the bacteria had destroyed, so the free spore was the only thing appeared. Though the spore was still intact and was still able to grow and recolonize on the AP after incubated.

The death process of bacteria and its spore

on the autoclave occurred in denaturation and protein coagulation inside the microbe cells.[18] Vapors produced as the sterilization process took place resulted in humid environment and forced all of the spores to germinate. In the heat and humid environment, the protein will be denaturated immediately, and subsequently the protein coagulation process took place and the bacteria died.[23] Based on this result, it is proved that the autoclave initiated on temperature of 121°C for 15 minutes is the ideal and qualified sterilization process to be used in critical sterile tools, and will be needed on aseptical work environment.

Also, after the oven and ozone sterilization for the 2nd (20-25 minutes), 3rd (30-35 minutes), 4th (40-45 minutes), and 5th (50-55 minutes), even though there were no live vegetative form, after it was incubated in 37 °C for 18-24 hours, the *Bacillus atrophaeus* spore was still able to colonize on AP culture. So, it can be concluded that the sterilization using the oven with ozone was only to kill the vegetative form, it was the disinfection process, even after repeated for 1, 2, 3, 4 and 5 times, still could not kill all the spores, or in other word it is only bactericide and not sporicide.[7]

Otherwise, in the oven and infrared sterilization decreased number of *Bacillus atrophaeus* spores relatively more than the oven and ozone sterilization. Infrared produced heat so that the temperature was higher. This heating will caused the spore initiation. So, at the next heating the vegetative form as a result of spore germination will be dead. That is why in the oven and infrared sterilization at the 2nd times (20th -25th minute) the number of spores grown on the AP culture were reduced than the 1st time (15 minutes). Also with the 3rd heating sterilization the spores would be reduced to 0 because more of them dead. This could explain why after the 3rd repetition (30th-35th minute) after the spores were dead, though it was unfortunate that the temperature could not be noted. After the 4th sterilization (40th-45th minute), and 5th (50th-55th minute), it seemed that all of the spores were missing from the micrograph and did not grown on the AP culture, which meant that all the spores were dead. Thus the heating processes by oven and infrared after the 3rd repetition could be called sterilization.

Based on this result, it was proved that oven equipped with infrared could be used for sterilization by repeating the process of heating up to minimally 3 times (30th-35th minute). The heat mechanism for destroying the bacterial spores which was through the protein, calcium, dipicolinate acid and DNA inactivation by absorbing all the water inside the cell protoplasm. That is why the spore dehydration occurred and the metabolism process stopped and resulted in cell death.[11]

Besides the heat also denaturated protein which mean the enzyme chemical bond for maintaining the natural enzime form had weakened. The destroyed chemical bond had resulted in the enzyme form distortion so that the enzyme was not functioning anymore and stopped the metabolism reaction which in time resulted in cell death.[9]

In autoclave, the temperature monitoring was easy. It was based on the number showed on the thermometer. Otherwise, the oven used in this study was not equipped with thermometer so it was difficult to monitor the temperature. For the better result, it was suggested that every manufacturer to equip the oven with the thermometer which could be easily read while the sterilization process initiated.

The sterilization process repetition at the 2nd, and 3rd in the oven and infrared, or in the 5th times at the oven and ozone could increased the temperature and sterilization time though after 5 minutes the "on" button turned to "off". It can be stated that the repetition of the processes means the heat increased so that the temperature and time also increased. In the 1st heating (15 minutes) generally bacteria had been dead and the initiation process occurred on the bacterial spores. That is why on the 2nd heating (20th-25th minute), the dead spores will be increased, so that all of the spores died at the 3rd heating (30th-35th minute).

It was different with the ozone, although the sterilization process repeated up to 5 times (50th-55th minute), there were still living spores, though all the vegetative form of bacteria had been dead. This sterilization process is good for food so that it turned dead all the vegetative form. If the contaminant were in small amount bacteria, it could be stated as sterile. That is why before sterilization all the tools had to be washed clean so the contaminant bacteria were in small amount.

CONCLUSION

Based on this study, it can be suggested that the bacterial spores usage as a biological indicator in tools and materials for odontectomy sterilization, to meet the requirements of standard precautions. If in the sterilization process with the oven+infrared achieved the sterile condition then the oven can be recommended in another sterilization process for another surgery procedures.

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