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Betalactamase Enzyme Role in Minimizing False-Positive Result of Cefotaxime Injection End-Product Sterility

Sri Agung Fitri Kusuma[1]*, Yus Hargono CY [2], Hendro Winarno[2]

[1]Department of Biology Pharmacy, Faculty of Pharmacy, Padjadjaran University, Sumedang, West Java, Indonesia 45363, [2]Department Of Pharmacy, Al-Ghifari University, Bandung, West Java, Indonesia

Abstract

Aim: The aim of this study was was to ensure quality assurance of cefotaxime injection sterility test by the addition of the betalactamase enzyme.

Methods: The cefotaxime injection product was sterilized using open filtration systems, then tested its sterility using standardized method modified by the addition of betalactamase enzyme with varying concentration in the sterility test medium. As a comparative result, the contaminated cefotaxime injection was tested with and without betalactamase enzyme addition. For sterility test, *Staphylococcus aureus* ATCC®6538, *Escherichia coli* ATCC®8739 and *Candida albicans* ATCC®10231 were used as test microbes with Fluid Tetrathionate Medium (FTM), Trypticase Soy Broth (TSB), Sabouraud Dextrose Agar (SDA), Pepton, Antibiotic Medium-1 (AM-1) and Tryptone Soya Agar (TSA) as the growth medium. The ambiguous contamination analysis was eliminated by cefotaxime breaking using betalactamase enzyme and the cefotaxime breakdown was confirmed by determining its antibacterial potential value using the agar diffusion method.

Results: The sterility test of sterile cefotaxime injection with the addition of the betalactamase enzyme gave sterile results and the loss of cefotaxime antibiotic potency. This suggested that the betalactamase enzyme breakdown the cefotaxime structure. This correlated with the sterile result of the contaminated cefotaxime injection with betalactamase addition, resulting the non sterile product. In contrast, sterility test resulted from contaminated injection product without belactamase addition, resulting in a false-negative result in the presence of antibiotic action against the contaminants.

Conclusion: The addition of betalactamase enzyme is needed to ensure the sterility of betalactam antibiotic-containing injectable product.

Keywords: betalactamase, enzymes, cefotaxime, false-negative, sterility

INTRODUCTION

Antibiotics are actually used to treat infections, but it can be a factor that endangers humans. Especially the sterile products of antibiotics in the form of antibiotic injection, which the route of drug administration directly enters the circulatory system. Recently, a news reported that the FDA has recalled an antibiotic containing injection drug, which mold was found in it. In another study, it has been reported that there has been contamination of *Burkholderia cepacia* in injection drugs containing vancomycin, thus resulting in patients experiencing hypoxia, hypotensive, and unresponsive with seizure activity, within 1 h after administering [1]. Infection with certain organisms should alert the health care provider to the possibility of contamination of intravenous products. Therefore, the pharmaceutical industry must consider the microbial contamination of parenteral products as one of the potential health problems that must be controlled. The contaminated parenteral product because of non-compliance to the rules of pharmaceutical products manufacture, attributed to the increased of hospitalizations number and death rates [2]. Therefore, the technique of sterilization becomes the main thing in assuring the quality of parenteral products. But it must be emphasized, that a pharmaceutical manufacturer must ensure that the sterility of parenteral products containing antibiotics is completely sterile by sterilized process, not because of antibiotic that contain in its formula. Because even though the antibiotic used in the formula produce their antimicrobial activity, but it is not purposed to self-sterilizing the product, in order to avoid contamination. Thus, in manufacturing of antibiotics-containing injectable drug, pharmacists must employ end-product sterility testing of antibiotics-containing injectable drug to ensure its sterility and prevent false negative results.

In this study, cefotaxime containing injectable drug was observed and analysis. It was associated with the discovery of cefotaxime dry injection preparations from one of distributor indicating that the preparation is not sterile, so that a sterility testing of the product must be performed. Whereas cefotaxime is a new third generation of semisynthetic cephalosporin which is administered intramuscularly and intravenously that has a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, also enterobacteriaceae multidrug-resistant. Generally, cefotaxime is more active against Gram-negative bacteria than the another generation of cephalosporins [3, 4]. Infection treatment with cefotaxime now exceeds 3000 cases, especially in septicemia, lower respiratory tract infections, meningitis, and pediatrics [4]. The most important correlation with end-product monitoring of injection cefotaxime is its ability to inhibit the growth of enterobacteriaceae multidrug-resistant bacteria. This can outwit the sterility test results of this injection product. Cefotaxime is thought to be able to self-sterilize in its injectable products. But, in the other hand, several bacteria has developed a resistance to third generation cephalosporins because of extended spectrum β-lactamase (ESBL) enzymes [5]. Thus, although cefotaxime has a wide spectrum, it does not guarantee that cefotaxime is capable of inhibiting all contaminants that contaminate the cefotaxime injection product. Therefore, sterility of the end product of cefotaxime injection should be performed.

To ensure the sterility of cefotaxime injection, sterility testing is employed. Membrane filtration is the method of choice for sterility testing of many antimicrobial-containing injectable solutions [6]. The sterility testing for detecting microbial contaminants in parenteral drugs methods using membrane filtration, as described in USP, are based on the media turbidity observation due to the growth of microbial contaminants. But the potential antibacterial of cefotaxime can disturb the result analysis. Therefore, the inhibitory effects of cefotaxime must be eliminated by dilution or by deactivation with chemical or enzymatic agents. Thus, to ensure the sterility test for cefotaxime, the test could be modified with betalactamase enzymes in addition to end product microbial contaminant testing. The enzyme has the extended spectrum β-lactamase against betalactam antibiotic including cephalosphorin [7].

MATERIALS AND METHODS

Materials

The chemicals used are the betalactamase enzyme, cefotaxime injection (sample), barium chloride and sulfuric acid. The growth media used in this study were Fluid Tetrathionate Medium (FTM), Tryptone Soya Agar (TSA), Trypticase Soy Broth (TSB),

Sabouraud Dextrose Agar (SDA), Pepton, and Antibiotic Medium-1 (AM-1). The tested microbes used in this study were *Staphylococcus aureus* ATCC®6538, *Escherichia coli* ATCC®8739, and *Candida albicans* ATCC®10231.

Medium Preparation

Preparation of agar media (FTM, TSA, TSB, SDA, Antibiotics Medium-1, and Pepton) used in this study was basically the same preparation procedure. The procedure step was weighed in a number of media (according to the Appendix of the medium bottle), and dissolved in 500 mL aquadest in 1000 beaker glass then heated to the hot plate and stirred until it dissolved. The media solution was then sterilized in the autoclave.

Media Fertility Test

The fertility test is used to know that the used media do not contain substances that can inhibit the growth of microorganisms. The test was performed by inoculating the indicator microbes into the medium and then incubated for 7 d at 30-35 °C for bacterial growth and at 20-25 °C for fungal growth. The bacterial indicator was *Bacillus subtilis*, while the indicator for fungal growth media was *Candida albicans*. This fertility test was performed after the standard dilution of bacterial colonies for fertility testing (*Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*), ie (10^{7}), in a pipette of 1 mL was added to petri and the media was added to total of 5 mL tested medium (such media: FTM, TSB, SDA, TSA, Pepton). The media then incubated at a temperature of 20 - 25^{o}C for 5 to observe the fungal growth and temperature 30 - 35^{o}C for 2 d to observe the bacterial growth.

Preparation of β-lactamase Enzyme Concentration

The determination of β-lactamase enzyme concentration was purposed to to find the volume standard of β-lactamase enzyme to be used for sterility test. The concentration of the beta-lactamase enzyme were prepared by taking a vial in the package of beta-lactamase enzyme (Lactamator: >100 IU cephalosporinase and >1000 IU penase / vial) and pipeted for 0.3; 0.7; 1.0 and 1.5 mL, then added to the 20 ml of FTM and TSB medium. Thus the tested concentrations were 1.5; 3.5; 5 and 7.5% v/v. After that, each of these concentrations was tested for the potential of antimicrobial activity. After the standard volume was obtained, further sterility testing was performed.

Potency Test

A total of 20 mL of MHA medium (temperature 40-45 ° C) was poured into a sterile petri dish, homogenized and left at room temperature to solidy (called base layer). This test was used 15 pieces of the sterile petri dish. Furthermore, microbial suspension was made using 0.5 standard Mac Farland. In a sterile Erlenmeyer, 100 mL of sterile media (mixture of 40-45 ° C) is mixed with a certain amount (0.5-2 mL) of microbial suspension and homogenized. A total of 10 mL of the test medium is poured into each sterile petri dish that has a base layer (called a seed layer). On the test media, holes were made using a stainless steel punch hole (diameter 6 mm) with a symmetrical distance of each hole ± 28-30 mm. Each of these holes was filled with 0.07 mL standard solution (S) and test solution (U) with a 5 + 1 dosage arrangement, such that the hole contained the standard solution and test solution. The dosage treatment positions were made across so that no overlapping of inhibiting zone between high sample doses and standard high doses was observed. The test medium was then incubated at 32-35 ° C for 16-18 h. The diameter of the inhibit zones formed were measured using a caliper.

Sterility Test

A total of 20 vial dry cefotaxime injectable antibiotics was poured into a 500 ml Erlenmeyer tube filled with 300 mL peptone solution. After homogeneous test solution, filtration was done using vacuum. The tube in manifold I was used for the examination of antibiotic sterility without beta-lactamase, whereas the manifold tube II was used for examination with the addition of beta-lactamase, and the manifold tube III was used for the examination of the blanks. Each filter paper from the filtration result was divided into 2 parts (part I for examination of fungal contamination using TSB media, part II for bacteria examination using FTM media). FTM media were incubated at 30-35^{0}C for 14 d, while TSB medium was incubated at 20-25^{o}C for 14 d.

RESULTS AND DISCUSSION

Bacterial Standard Concentration

The preparation of this standard of bacterial concentration was done as a first step to test the fertility of the media. The results showed that the concentration of *S. aureus* (10^{-7}) with the average number of selective colonies of the SNC (Small Number Concentration) was 89, *E. coli* (10^{-6}) with the average number of selective colonies of SNC was 70, meanwhile *C. albicans* (10^{-6}) with the number of selective colonies of the SNC was 60. Result of media fertility test could be seen in table 1.

Media Fertility Test Results

This test is important to ensure its ability in supporting microbial growth. The achieving of accurate and reproducible microbiological test results will be obtained by the using of high quality media [8]. The results of media fertility tests include peptone media, FTM, TSB, SDA, TSA and Antibiotics Medium-1, shown in Table 2-7.

Table 1: Bacterial Concentration

Microbes	Numbers of colonies in each dilution							Dilutions that meet the SNC specifications	Average colony	note
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}			
S. aureus	~	~	~	~	~	~	97	10^{-7}	89	passed
	~	~	~	~	~	~	81			
E. coli	~	~	~	~	~	62	0	10^{-6}	70	passed
	~	~	~	~	~	78	0			
C. albicans	~	~	~	~	~	63	42	10^{-6}	60	passed
	~	~	~	~	~	57	36			

Notes: (~) countless; SNC (Small Number Concentration)

Table 2: Fertility Test Of Pepton Medium

Microbes	Cfu test	Cfu control	% recovery ((cfu test:cfu control) x 100%)	% recovery spesification	Note
S. aureus	80	89	89.9	≥ 80%	passed
E. coli	52	60	86.7	≥ 80%	passed
C. albicans	64	70	91.4	≥ 80%	passed

Table 3: Fertility Test Of FTM Medium

Microbes	Cfu test	Cfu control	% recovery ((cfu test:cfu control) x 100%)	% recovery spesification	Note
S. aureus	85	89	95.5	≥ 80%	passed
E. coli	51	60	85.0	≥ 80%	passed
C. albicans	61	70	87.1	≥ 80%	passed

Table 4: Fertility Test Of TSB Medium

Microbes	Cfu test	Cfu control	% recovery ((cfu test:cfu control) x 100%)	% recovery spesification	Note
S. aureus	84	89	94.4	≥ 80%	passed
E. coli	54	60	90.0	≥ 80%	passed
C. albicans	62	70	88.6	≥ 80%	passed

Table 5: Fertility Test Of SDA Medium

Microbes	Cfu test	Cfu control	% recovery ((cfu test:cfu control) x 100%)	% recovery spesification	Note
S. aureus	84	89	94.4	≥ 80%	passed
E. coli	55	60	91.7	≥ 80%	passed
C. albicans	68	70	97.1	≥ 80%	passed

Table 6: Fertility Test Of TSA Medium

Microbes	Cfu test	Cfu control	% recovery ((cfu test:cfu control) x 100%)	% recovery spesification	Note
S. aureus	84	80	89.9	≥ 80%	passed
E. coli	55	55	91.7	≥ 80%	passed
C. albicans	68	70	92.9	≥ 80%	passed

Table 7: Fertility Test Of Antibiotics Medium-1

Microbes	Cfu test	Cfu control	% recovery ((cfu test:cfu control) x 100%)	% recovery spesification	Note
S. aureus	83	80	93.6	≥ 80%	passed
E. coli	53	55	88.3	≥ 80%	passed
C. albicans	65	70	92.9	≥ 80%	passed

Based on the results of media fertility test, it was concluded that all media to be used in the sterility test of cefotaxime injection was stated to meet the requirement, because it showed above 80% recovery.

Table 8: Effect 1.5% Betalactamase Enzyme To Sterility Test

Incubation period (day)	Test medium		Negative control		Positive control	
	FTM	TSB	FTM	TSB	FTM	TSB
1	-	-	-	-	-	-
2	-	-	-	-	+	+
3	-	-	-	-	N/A	N/A
4	-	-	-	-	N/A	N/A
5	-	-	-	-	N/A	N/A
6	-	-	-	-	N/A	N/A
7	-	-	-	-	N/A	N/A
8	-	-	-	-	N/A	N/A
9	-	-	-	-	N/A	N/A
10	-	-	-	-	N/A	N/A
11	-	-	-	-	N/A	N/A
12	-	-	-	-	N/A	N/A
13	-	-	-	-	N/A	N/A
14	-	-	-	-	N/A	N/A

Determination of Betalactamase Enzyme Concentration

β-lactamase enzymes are the enzyme group that can cleave the amide bond in beta lactam rings of beta lactam antibiotics so that these enzymes rendering the antibiotic ineffective to bacteria and causing resistance. Cefotaxime sodium is one of a cephalosporin antibiotic, commonly use in parenteral form, performs its bactericidal activity by inhibiting the cell wall synthesis of bacterial [**9**]. One of the betalactamases reported can hydrolyzes cefotaxime with about 1000-fold higher catalytic efficiency [10, 11]. This mechanism can be utilized to inhibit cefotaxime antibacterial work on a sterilization test of injection products containing cefotaxime. Thus, the sterility of the product can be believed to originate from the correct process of manufacture and sterilization, not due to the antibiotic role against contaminants inside the injection product. Therefore, this study determined the concentration of betalactamase enzyme for the quality assurance of sterilization test results. The results were performed in table 8-11. The sterility test of cefotaxime injection using a varying concentration of betalactamase enzyme showed that in minimal concentration of the enzymes (1.5 %v/v) had inhibited the cefotaxime activity. Thus, the negative sterility result was the correct result that assures the quality of the injection product

Antibiotic Potency and Sterility Test of Contaminated Product

The antibiotic potency test must be conducted to measure the bioactivity, prior to market release. Through microbiological assay, the antibiotic ability to inhibit the target organisms, can be verified. Therefore, it is important to check the antibiotic potency result before formulating it. The antibiotic tests were done to the cefotaxime that contacted with betalactamase enzymes in varying concentration, to the pure cefotaxime (before formulated) and to the contaminated cefotaxime injection. The potency test result of cefotaxime that being contacted with betalactamase enzyme could be observed in figure 1.

.Table 9: Effect 3.5% Betalactamase Enzyme To Sterility Test

Incubation period (day)	Test medium		Negative control		Positive control	
	FTM	TSB	FTM	TSB	FTM	TSB
1	-	-	-	-	-	-
2	-	-	-	-	+	+
3	-	-	-	-	N/A	N/A
4	-	-	-	-	N/A	N/A
5	-	-	-	-	N/A	N/A
6	-	-	-	-	N/A	N/A
7	-	-	-	-	N/A	N/A
8	-	-	-	-	N/A	N/A
9	-	-	-	-	N/A	N/A
10	-	-	-	-	N/A	N/A
11	-	-	-	-	N/A	N/A
12	-	-	-	-	N/A	N/A
13	-	-	-	-	N/A	N/A
14	-	-	-	-	N/A	N/A

Table 10: Effect 5% Betalactamase Enzyme To Sterility Test

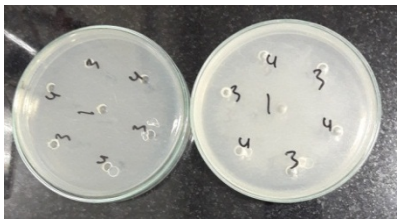
Incubation period (day)	Test medium		Negative control		Positive control	
	FTM	TSB	FTM	TSB	FTM	TSB
1	-	-	-	-	-	-
2	-	-	-	-	+	+
3	-	-	-	-	N/A	N/A
4	-	-	-	-	N/A	N/A
5	-	-	-	-	N/A	N/A
6	-	-	-	-	N/A	N/A
7	-	-	-	-	N/A	N/A
8	-	-	-	-	N/A	N/A
9	-	-	-	-	N/A	N/A
10	-	-	-	-	N/A	N/A
11	-	-	-	-	N/A	N/A
12	-	-	-	-	N/A	N/A
13	-	-	-	-	N/A	N/A
14	-	-	-	-	N/A	N/A

Table 11: Effect 7.5% Betalactamase Enzyme To Sterility Test

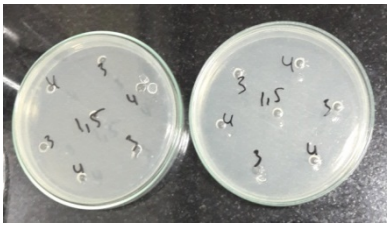
Incubation period (day)	Test medium		Negative control		Positive control	
	FTM	TSB	FTM	TSB	FTM	TSB
1	-	-	-	-	-	-
2	-	-	-	-	+	+
3	-	-	-	-	N/A	N/A
4	-	-	-	-	N/A	N/A
5	-	-	-	-	N/A	N/A
6	-	-	-	-	N/A	N/A
7	-	-	-	-	N/A	N/A
8	-	-	-	-	N/A	N/A
9	-	-	-	-	N/A	N/A
10	-	-	-	-	N/A	N/A
11	-	-	-	-	N/A	N/A
12	-	-	-	-	N/A	N/A
13	-	-	-	-	N/A	N/A
14	-	-	-	-	N/A	N/A

a. Inhibition of cefotaxime in 1.5% BE

b. b. Inhibition of cefotaxime in 3.5% BE



c. Inhibition of cefotaxime in 5.0% BE



d. d. Inhibition of cefotaxime in 7.5% BE

Figure 1: Cefotaxime potency result with betalactamase addition
Note: betalactamase enzyme (BE)

The results above showed that sterilization produced a guaranteed sterile product because the antibiotic cefotaxime had been successfully degraded by the enzyme betalactamase in the concentration variations used. In comparison with the effect of betalactamase enzyme addition to sterility quality, this study also conducted product exposure to contaminants. Thus, it could be proven the importance of betalactamase enzyme addition, to avoid false-positive result of cefotaxime injection end-product sterility.

The antibiotic potency of pure cefotaxime rendering the optimal potency value i.e. 100%. It means that the cefotaxime material used in this study had good quality. Meanwhile, the potency value of cefotaxime containing in contaminated injection product. Eventhough, the injectable product containing cefotaxime had been exposed to the contaminants, but the value of the antibiotic remains high i.e. 99,86%. This result was correlated with sterility test result of contaminated product with and without betalactamase enzyme addition, shown in table 11-12.

Based on the results of sterility test, the effect of betalactamase enzyme addition on the sterility test of the product showed a different sterility level. Supposedly, by exposure to contaminants, the media used in the sterility test, will be contaminated. Because the expected result was the quality of the product through a sterility test that was not biased with antimicrobial action of cefotaxime. However, this can only be known if cefotaxime was destroyed first using the betalactamase enzyme. The data in table 11, showing that injection products exposed by contaminants, exhibited false-negative results. This strongly suggested that

cefotaxime was potential as an antimicrobial for contaminants exposed to the preparation. The assumption was supported by the data in table 12, which showed that if the betalactamase enzyme was added to the sterilization process, then this enzyme will destroy the antimicrobial action of cefotaxime. Thus, the contaminated cefotaxime injection product showed non-sterile results. This showed the importance of the addition of the betalactamase enzyme as a detection test for the quality of sterile products.

Table 11: Result of Contaminated Injection Sterility Test Without Betalactamase Enzyme Addition

Incubation period (day)	Test medium		Negative control		Positive control	
	FTM	TSB	FTM	TSB	FTM	TSB
1	-	-	-	-	-	-
2	-	-	-	-	+	+
3	-	-	-	-	N/A	N/A
4	-	-	-	-	N/A	N/A
5	-	-	-	-	N/A	N/A
6	-	-	-	-	N/A	N/A
7	-	-	-	-	N/A	N/A
8	-	-	-	-	N/A	N/A
9	-	-	-	-	N/A	N/A
10	-	-	-	-	N/A	N/A
11	-	-	-	-	N/A	N/A
12	-	-	-	-	N/A	N/A
13	-	-	-	-	N/A	N/A
14	-	-	-	-	N/A	N/A

Table 12: Result of Contaminated Injection Sterility Test With Betalactamase Enzyme Addition

Incubation period (day)	Test medium		Negative control		Positive control	
	FTM	TSB	FTM	TSB	FTM	TSB
1	-	-	-	-	-	-
2	+	+	-	-	+	+
3	+	+	-	-	N/A	N/A
4	+	+	-	-	N/A	N/A
5	+	+	-	-	N/A	N/A
6	+	+	-	-	N/A	N/A
7	+	+	-	-	N/A	N/A
8	+	+	-	-	N/A	N/A
9	+	+	-	-	N/A	N/A
10	+	+	-	-	N/A	N/A
11	+	+	-	-	N/A	N/A
12	+	+	-	-	N/A	N/A
13	+	+	-	-	N/A	N/A
14	+	+	-	-	N/A	N/A

Microbial contamination of parenteral products is one of the most serious issues currently facing the pharmaceutical industry. This could happen because of a lack of standardized methods for the contaminants detection [12, 13]. In the real condition, the antibiotic injectable product can be contaminated because of the postautoclave processing of the injection bottle and the screw-cap closure of the bottle [14**,** 15]. The outbreaks of septicemia because of the finding of Enterobacter cloacae or Enterobacter agglomerans were the example case caused by intrinsic microbial contamination of a newly introduced screw-cap closure [15, 16]. Most septicemias probably arose from the transfer of microorganisms present in the closure of fluid during therapy. Therefore, in addition to sequential sampling, other improvements in quality control are needed. Our results study can be used as an improvement sterility test method to avoid the false-negative result of the antibiotic sterile product.

CONCLUSION

The conclusion of this study is the addition of betalactamase enzyme in the sterility test process of betalactam antibiotic injection could improve the quality assurance of the end product sterility.

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