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Research Article

In vitro mutant prevention concentration of colistin sulfate against pathogenic *Escherichia coli*

[1,2]Maria F. Palupi, [3,5]Huda S. Darusman, [3]Hera Maheshwari, [4]I Wayan T. Wibawan, [4]Etih Sudarnika

[1] National Veterinary Drug Assay Laboratory, Directorate General of Livestock and Animal Health-Ministry of Agriculture of the Republic of Indonesia; [2] Physiology and Pharmacology Study Program, Graduate School of Bogor Agricultural University, Indonesia; [3] Department of Anatomy, Physiology, and Pharmacology, Veterinary Medicine Faculty, Bogor Agricultural University, Indonesia;[4] Department of Animal Disease and Veterinary Public Health, Veterinary Medicine Faculty, Bogor Agricultural University, Indonesia; [5] Primate Research Center, Bogor Agricultural University, Indonesia.

Abstract. Mutant prevention concentration (MPC) is an in vitro measurement to determine the lowest drug concentration to inhibit the growth of all single-step-mutant subpopulations. The objective of this research is to study the MPC value of colistin sulfate alone and in combination with amoxicillin trihydrate to prevent the growth of mutant subpopulation from susceptible *Escherichia coli* pathogen isolates. The MPC value was then determined by the agar dilution method. Mueller-Hinton agar containing colistin standard alone and in combination with amoxicillin was inoculated with 10^{10} cfu *E. coli*. The MPC value of colistin sulfate alone against 18 pathogen-susceptible colistin *E. coli* isolates was between 16-128 μg mL^{-1} (41.78 ± 25.23 μg mL^{-1}) that give mutant selection windows range between 1.36 ± 0.62 μg mL^{-1} and 41.78 ± 25.23 μg mL^{-1}. Two pathogen *E. coli* isolates susceptible to colistin and amoxicillin, coded K14d and K14e, were used to determine the MPC value of colistin sulfate in combination with amoxicillin trihydrate. The MPC value of colistin sulfate-amoxicillin trihydrate combination was obtained in the concentration of colistin 16 μg mL^{-1} (code K14d) and 2 μg mL^{-1} (code K14e) with the concentration of amoxicillin 512 μg mL-1 for both isolates. Combining colistin sulfate with very high amoxicillin trihydrate concentration can reduce the MPC value of colistin sulfate. According to this result, we urge to reduce the usage of colistin sulfate in animal production.

Key Words: Mutant prevention concentration, colistin, *Escherichia coli*

Corresponding Author: M. F. Palupi, email: lupi_ima@yahoo.co.id

Introduction

Colistin sulfate is one of the antimicrobial agents that are used in animals and humans. Colistin sulfate is a polymyxin antibiotic and has been used as the last choice of drug against the infection of gram negative pathogen multidrug-resistant (MDR) carbapenemase bacteria (Falagas & Kasiakou 2005; Collignon et al 2009; Catry et al 2015; Nordmann et al 2016). In general, the usage of colistin sulfate in humans is to treat bacterial infections topically or orally. However, the usage of colistin sulfate has diminished due to its significant nephrotoxicity and neurotoxicity (Falagas & Kasiakou 2005; Morales et al 2012). Colistin sulfate has been widely used in food animals for the treatment of infectious diseases caused by gram negative bacteria such as *Salmonella, Pasteurella, Enterobacter*, and *E. coli* (Catry et al 2015; Pyorala et al 2015). Therefore, colistin has an important role for humans and animals. The discovery of the same colistin resistant *E. coli* isolates in both animals and humans shows the possibility of transferring them from animals to humans (Olaitan et al 2015). Food animals and their environment are considered as the important factors for the spread of resistant bacteria in humans. One of the bacteria used as the parameter of resistance monitoring in animals is *E. coli* (OIE 2016). The cases of antimicrobial resistance – especially antibiotic resistance – tend to increase globally. The used therapeutic concentration becomes one of the causative entities to the development of resistance. The concentration determined from the minimum inhibitory concentration (MIC) value is able to block or inhibit the growth of susceptible pathogens; however, it also selectively enriches the resistant mutant (Gebru et al 2011). The MIC value is still very useful to determine the susceptibility of antimicrobial, but it cannot be used to determine the true dynamics of high-density bacterial infection. The test of MIC using the micro dilution method with 10^5 cfu mL^{-1} inoculums or 10^4 cfu per spot by using agar dilution method, cannot be used to detect the development of resistance subpopulation in the 10^6-10^8 cfu or more bacteria population (Blondeau 2009; Hindler & Humphries 2013).

The hypothesis of mutant selection window (MSW) or drug concentration between the MIC value and the mutant prevention concentration (MPC) value has been introduced as one of the new strategies to investigate the development of antimicrobial resistance. Within the range of MSW concentration, the growth of susceptible cells is likely inhibited, but the growth of mutant cells will not be inhibited – which is known as a single-step mutation. The MPC defined by Mouton et al (2005), can be used to measure the potency of antimicrobial and to predict the resistance selection during the treatment of patients (Choi & Ko et al 2014).

In order to prevent the presence of resistant bacteria due to a single step mutation related to the administration of antibiotics, the MIC, MPC, and MSW data are needed. The MPC data of colistin sulfate against *E. coli* can be used as a reference to consider the continuity the usage of colistin sulfate in food animals in accordance with the development of resistance. The aim of this research is to determine the MPC value of colistin sulfate against *E. coli* pathogen isolates that were collected from the cloacal swabs of broilers in Indonesia.

Materials and methods

Selection of *Escherichia coli* isolates and mutant prevention concentration test of colistin sulfate alone

This study was conducted from October 2017 to February 2018 at the National Veterinary Drug Assay Laboratory (NVDAL), the Directorate General of Livestock and Animal Health, the Ministry of Agriculture of the Republic of Indonesia. A total of 235 *E. coli* isolates were obtained from the samples taken from the cloacal swab of broiler from 47 commercial broiler flocks in Bogor Regency, West Java Province - Indonesia. In vitro MPC test can only be used in susceptible colonies or isolates. This test is not effectively done by using bacteria that have resistant genes (Blondeau 2009). For our study, the selected isolates to determining the MPC value of colistin sulfate alone must be susceptible to colistin sulfate and pathogen. All of the isolates were tested for their susceptibility to colistin sulfate using the agar dilution method (Bahera et al 2010; Morales et al 2012). All isolates were inoculated to Mueller-Hinton agar (MHA, Oxoid-UK) containing colistin sulfate standard (Sigma-USA) with two-fold concentration dilution ranging from 0.125 μg mL^{-1} to 16 μg mL^{-1} and were incubated at the temperature of 37ºC for 24 hours. *Escherichia coli* ATCC 25922 was used as the controlling isolate, while MHA without colistin sulfate was used as the controlling media. The isolates were considered susceptible to colistin when their MIC value is < 2 μg mL^{-1} (Morales et al 2012; EUCAST 2017). To determine the pathogenic *E. coli* isolates, all samples were tested using Congo-Red test (Berkhoff & Vinal 1986). The susceptibility and Congo-Red test for each isolate was repeated three times.

To determine the MPC value of colistin sulfate alone, pathogen and susceptible colistin *E. coli* isolates were inoculated to 3 petri discs of Heart Infusion Agar (HIA, DB/Difco-FRA) and all were incubated for 18-24 hours at 37ºC. All contents of inoculated petri discs were removed with a sterile cotton swab and transferred into a tube containing 100 mL Mueller-Hinton Broth (MHB, DB/Difco-FRA). Inoculated MHB was then incubated for 18-24 hours at 37ºC. After the incubation, the inoculated MHB was centrifuged at 5000 x g for 30 minutes at 4ºC. Pellet was then re-suspended with 3 mL MHB. The MPC value was determined for inoculating 100 μL of MHB or equal to 10^{10} cfu *E. coli* to MHA which contains colistin sulfate standard (Sigma-USA) with two-fold concentration dilution ranging from 0.5 μg mL^{-1} to 128 μg mL^{-1} and was then incubated for 24 hours at 37ºC. If there were still isolates grown in MHA with 128 μg mL^{-1} colistin sulfate, the procedures were then repeated using MHA with a higher colistin sulfate concentration until the concentration that is able to prevent the growth of isolate was reached (Blondeau 2009; Gebru et al 2011; Nordqvist et al 2016).

Selection of *Escherichia coli* isolates and mutant prevention concentration test colistin sulfate combined with amoxicillin trihydrate

All isolates that had been selected for the MPC colistin sulfate alone test were then tested for their susceptibility for amoxicillin. The susceptibility tests of amoxicillin were conducted using the agar dilution method. The MHA media containing amoxicillin trihydrate standard (Sigma-USA) with twofold concentration dilution ranging from 0.5 μg mL^{-1} to 128 μg mL^{-1}. *Escherichia coli* isolates were considered susceptible to amoxicillin when their MIC value is < 32 μg mL^{-1} (Abu-Basha et al 2012).

The determination of the MPC value of colistin sulfate combined with amoxicillin trihydrate was using pathogen and colistin-amoxicillin susceptible isolates. The selected *E. coli* isolates were inoculated to 3 petri discs of HIA and all were incubated for 18-24 hours at 37ºC. The next procedure to reach a higher density of *E. coli* was conducted with the same method described previously. The MPC value was determined for inoculating 100 μL of MHB or equal to 10^{10} cfu *E. coli* to MHA which contains colistin sulfate standard (Sigma-USA) with two-fold concentration dilution ranging from 0.5 μg mL^{-1} to 16 μg mL^{-1} combining with amoxicillin trihydrate standard with twofold concentration from 2 μg mL^{-1} to 512 μg mL^{-}1. The MPC value from this combination was obtained from the combination of colistin sulfate and amoxicillin trihydrate that was able to prevent the growth of inoculation 10^{10} cfu *E. coli*. The MPC test was repeated three times.

Results

Selection of *Escherichia coli* isolates and mutant prevention concentration test colistin sulfate alone

Based on the results of the susceptible test of colistin sulfate and Congo Red test of 235 *E. coli* isolates, 18 candidates of *E. coli* pathogen isolates and susceptible colistin sulfate with MIC value between 0.5 μg mL^{-1} - 2 μg mL^{-1} were obtained. The 18 isolates were then tested for their MPC values. The MPC values obtained were between 16 μg mL^{-1} - 128 μg mL^{-1}. One isolate (5.6%) had MPC value of 16 μg mL^{-1}, 13 isolates (72.3%) had MPC value of 32 μg mL^{-1}, 3 isolates had MPC value of 64 μg mL^{-1}, and 1 isolate (5.6%) had MPC value of 128 μg mL^{-1}. The MPC values of all *E. coli* isolates tested were above the colistin resistance value of > 2 μg mL^{-1} based on MIC values. The results of MIC, MPC, and Mutant Prevention Index (MPI) or the ratio between MIC and MPC are recorded in Table 1.

The results in Table 1 show that the MPI values range from 8-128, whereas the MPC values of isolates range from 8-128 times out of those of MIC. In this study, the lowest MPI value was 8, which was from *E. coli* K45c isolate. This MPI value was still high because it took 8 times of the dose of MIC to prevent the growth of bacteria from K45c isolate into a mutant or resistance to colistin. Based on the test results obtained, the range of MSW varied from the lowest boundary using MIC value of 0.5 μg mL^{-1} to the highest boundary using MPC value of 128 μg mL^{-1}. Therefore, in this study, a further MPC test was performed using a combination of colistin sulfate-amoxicillin trihydrate to decrease the value of MPC colistin sulfate. The selection of colistin sulfate-amoxicillin trihydrate combination in this study was based on the most common combination registered in Indonesia (DGLAH 2016).

Table 1 MIC, MPC, and MPI value of colistin sulfate against *Escherichia coli*

No.	Code of *E. coli*	MIC (μg mL^{-1})	MPC (μg mL^{-1})	MPI
1	K19c	2	32	16
2	K25b	1	32	32
3	K26b	0.5	32	64
4	K27a	2	32	16
5	K32c	1	128	128
6	K34c	1	64	64
7	K35c	2	32	16
8	K35e	2	32	16
9	K41b	1	32	32
10	K45c	2	16	8
11	K45e	2	32	16
12	K54a	1	64	64
13	K14d	0.5	32	64
14	K14e	1	32	32
15	K26e	2	64	32
16	K27c	0.5	32	64
17	K14b	2	32	16
18	K37c	1	32	32

Selection of *Escherichia coli* isolates and mutant prevention concentration test colistin sulfate combined with amoxicillin trihydrate

All 18 isolates used for an MPC value of colistin sulfate alone were tested for their susceptibility to amoxicillin trihydrate and only two isolates were susceptible to amoxicillin trihydrate. The *E. coli* isolates which were susceptible to colistin sulfate and amoxicillin trihydrate were those coded K14d and K14e. The MIC value of amoxicillin trihydrate for both isolates was 1 μg mL^{-1}.

The MPC test of colistin sulfate-amoxicillin trihydrate was carried out using the agar dilution method using MHA containing both antibiotics with a standard combination of colistin sulfate with twofold concentration dilution from 0.5 μg mL^{-1} to 16 μg mL^{-1} and amoxicillin trihydrate with twofold concentration dilution from 2 μg mL^{-1} up to 512 μg mL^{-1}. There were 60 combinations of colistin sulfate-amoxicillin trihydrate concentration. The value of MPC combinations of colistin sulfate-amoxicillin trihydrate against *E. coli* K14d was 16 μg mL^{-1} colistin sulfate and 512 μg mL^{-1} amoxicillin. Meanwhile, for *E. coli* K14e, the value of MPC combinations obtained was 2 μg mL^{-1} colistin sulfate and 512 μg mL^{-1} amoxicillin. The MPC test of combining colistin sulfate-amoxicillin trihydrate was repeated three times and showed the consistent value as presented in Table 2.

Discussions

The MPC is a pharmacodynamic parameter that can be very useful in determining the dosage of antimicrobial used to prevent the emergence of a single-step bacterial resistance mutant against it. The MPC value of colistin sulfate alone against 18 pathogen-susceptible colistin *E. coli* isolates was between 16-128 μg mL^{-1} (41.78 ± 25.23 μg mL^{-1}) that give mutant selection windows range between 1.36 ± 0.62 μg mL^{-1} and 41.78 ± 25.23 μg mL^{-1}. The results of the MPC values from this study were then compared with pharmacokinetic data of colistin sulfate on broilers that were studied by Lashev and Haritova (2003). Pharmacokinetic data were obtained from the broilers that were given a dose of 50 mg colistin sulfate per kg BW by per oral administration. Based on the pharmacokinetics data, the maximum concentration (Cmax) of colistin in serum was 6.13 ± 0.44 μg mL^{-1}, the minimum concentration (Cmin) was 1.54 ± 0.22 μg mL^{-1}, and the concentration of area under the curve (AUC) was 18.75 ± 4.56 μg.h mL^{-1}. The concentration of colistin sulfate found in serum 4 hours after the administration of the repeated oral doses (50 mg colistin sulfate per L drinking water) for 5 days ranged from 0.73 ± 0.15 to 2.09 ± 0.22 μg mL^{-1} (Lashev & Haritova 2003).

The pharmacokinetic data obtained from the study conducted by Lashev and Haritova (2003) show that the concentration of colistin sulfate in blood and the AUC value are within the MSW range of colistin sulfate alone in this study. If the concentration is still within the MSW value, the subpopulation of mutant resistance can be developed (Cai et al 2010). Colistin is an antimicrobial that has pharmacokinetic and pharmacodynamic characteristics using the ratio of AUC and MIC in determining antibacterial activities and inhibiting the rate of resistance (Blondeau 2009). In order to provide satisfactory treatment effects and to reduce the appearance of resistant bacteria during treatment, the expected AUC/MIC ratio should be > 125 (Blondeau, 2009). Using the AUC value from the study done by Lashev and Haritova (2003) and MIC from our study, the AUC/MIC ratio of this study was vary between 9.4 - 37.5 (17.71 ± 10.11) or lower than the expected ratio.

As showed on the results of the MPC test of colistin sulfate-amoxicillin trihydrate, the value of MPC colistin sulfate in both *E. coli* K14d and K14e were lower from the MPC value of colistin sulfate alone. With the declining of MPC value, giving colistin sulfate combined with amoxicillin trihydrate has made the MSW range of colistin sulfate become narrower compared with colistin sulfate alone. The MPC colistin sulfate value in *E. coli* K14d decreased from 32 μg mL^{-1} to 16 μg mL^{-1}, while in *E. coli* K14e it decreased from 32 μg/ mL^{-1} to 2 μg mL^{-1}. Using the MPC value of colistin sulfate in a combination form, the MPI value of colistin sulfate with *E. coli* K14d decreased from 64 to 32. The value of MPI colistin sulfate of *E. coli* K14e decreased drastically from 32 to 2. The range of MSW obtained from MIC and MPC of colistin sulfate alone against *E. coli* K14d isolate was 0.5-32 μg mL^{-1}. Meanwhile, in combination with amoxicillin trihydrate, the range of MSW dropped to 0.5-16 μg mL^{-1}. The MSW range of the single colistin sulfate for *E. coli* K14e was between 1-32 μg mL^{-1}, while in combination with amoxicillin trihydrate the MSW range dropped to 1-2 μg mL^{-1}. Based on the MPC value of colistin sulfate-amoxicillin trihydrate obtained by using *E. coli* K14d, the concentration of colistin sulfate in blood and the AUC from Lashev and Haritova (2003) study were still within the MSW range of colistin. Using the MPC value obtained from the combinations of colistin sulfate-amoxicillin trihydrate for *E. coli* K14e, the concentration of colistin sulfate in the blood and the AUC at a dose of 50 mg

Table 2 The results of MPC test of combined colistin sulfate-amoxicillin trihydrate *Escherichia coli* code K14d and K14e

Concentration of amoxicillin trihydrate	***Escherichia coli* K14d**						***Escherichia coli* K14e**					
(μg mL^{-1})	Concentration of colistin sulfate (μg mL^{-1})						Concentration of colistin sulfate (μg mL^{-1})					
	0.5	1	2	4	8	16	0.5	1	2	4	8	16
2	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	+
64	+	+	+	+	+	+	+	+	+	+	+	+
128	+	+	+	+	+	+	+	+	+	+	+	+
256	+	+	+	+	+	+	+	+	+	+	+	+
512	+	+	+	+	+	-	+	+	-	-	-	-

kg^{-1} BW were above MSW range or reached the MPC value. A combination with amoxicillin, successfully narrower the MSW range for *E. coli* K14e until 16 times compared giving colistin sulfate alone. However, the MPC value of amoxicillin trihydrate for both isolates was very high – it reached 512 μg mL^{-1}. The MIC value of amoxicillin trihydrate for both isolates was 1 μg mL^{-1}, so the MPI value of amoxicillin trihydrate obtained for both isolates was 512. The MSW range of amoxicillin trihydrate for *E. coli* K14d and K14e was 1 -512 μg mL^{-1}.

Using the pharmacokinetic data of amoxicillin in broilers from the study conducted by Werdiningsih et al (2008), the Cmax of amoxicillin in blood was 3.77-5.04 μg mL^{-1}. The given dose was 20 mg per kg BW. The concentration of amoxicillin trihydrate in the blood was still within the range of MSW value of amoxicillin of *E. coli* K14d and K14e. Therefore, in order to block the occurrence of single step mutant colistin resistance from *E. coli* K14d and K14e, a combination with high doses of colistin sulfate and amoxicillin trihydrate is required. Extremely higher doses may not be applicable because those can lead to undesirable side effects, excessive residue, increased medical expenses, and drug contamination in flock environment.

The MPC approach can be used as an alternative method in the effort to reduce the occurrence of resistant bacteria caused by a single step mutant from the subpopulation of resistant bacteria. From this MPC study, we can determine the MSW and MPI of colistin sulfate that very important to seek the possibility of the emergence of single step mutation from the dosage that given. The results of this study showed the MPC of colistin sulfate alone was between 16 – 128 μg mL^{-1} (41.78 ± 25.23 μg mL^{-1}). As a result of these high MPC value, giving consequences a wide MSW range of colistin sulfate. The wide MSW range also supported by the result of MPI value. The MPI colistin sulfate against *E. coli* value varied between 8-128 (39.56 ± 30.12). This MPI was higher than the MPI of doripenem, ertapenem, imipenem, and meropenem against *E. coli* (Credito et al 2010). Meanwhile, to suppress the emergence of single step mutation we need lower MPI and narrower MSW range. The lower MPI value indicates a better ability to prevent the growth of resistance or a single step mutant (Credito et al 2010). Using AUC data from broiler that given colistin sulfate in high dosage, 50 mg/kg BB, the AUC value still within in MSW range. It showed that even given in high dosage of colistin, the subpopulation mutant or single-step mutation that resistance to colistin sulfate can still develop.

Our study also showed that giving colistin sulfate combined with amoxicillin can reduce the MPC colistin sulfate value. The range of colistin sulfate MSW from *E. coli* isolate K14d becomes narrower, but the emergence of single step mutations colistin resistance from isolate K14d can still occur because the AUC value is still in the MSW range. Meanwhile, the MSW range of colistin sulfate from *E. coli* isolate K14e dropped dramatically and the AUC value was above MPC, but the MSW range of amoxicillin was very wide. This means that even we can prohibit the emergence of a single colistin resistance mutation from isolate K14e but requires a very high dose of amoxicillin to achieve MPC value.

As we know, colistin sulfate plays an important role as the last drug choice in handling infection cases caused by pathogenic negative bacteria MDR in humans. Recently, the effectiveness of colistin sulfate as the last drug choice is increasingly threatened by the discovery of colistin-resistant genes that can be transferred via plasmids such as mcr-1 gene (Paterson & Harris 2016). Therefore the use of colistin sulfate in animal production must be reconsidered. Based on MIC and MPC data from this study against *E. coli* pathogens originating from broilers, has shown that there is a wide range of MSW that allows a single step mutation that can develop colistin resistance.

Conclusion

This study concludes that colistin sulfate has a high MPC value between 16 – 128 μg mL^{-1} (41.78 ± 25.23 μg mL^{-1}) with the consequence of wide range MSW. Therefore, based on the MPC, MSW, and MPI data we obtained from our study, we urge a reduction in the use of colistin sulfate in animal production to minimize the development of *E. coli* colistin resistance.

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Authors

•Maria Fatima Palupi, National Veterinary Drug Assay Laboratory, Directorate General of Livestock and Animal Health-Ministry of Agriculture of the Republic of Indonesia, Jalan Raya Pembangunan Gunungsindur, Bogor, West Java, Indonesia - 16340; Physiology and Pharmacology Study Program, Graduate School of Bogor Agricultural University; Jalan Agatis, Kampus IPB Dramaga, Bogor, West Java, Indonesia - 16880; email: lupi_ima@yahoo.co.id

•Huda Salahuddin Darusman, Department of Anatomy, Physiology, and Pharmacology - Veterinary Medicine Faculty, Bogor Agricultural University, Jalan Agatis, Kampus IPB Dramaga, Bogor, West Java, Indonesia - 16880; Primate Research Center, Bogor Agricultural University, Jalan Lodaya II, Bogor, West Java, Indonesia - 16128; email: hudauppsala@yahoo.com

•Hera Maheshwari, Department of Anatomy, Physiology, and Pharmacology - Veterinary Medicine Faculty, Bogor Agricultural University, Jalan Agatis, Kampus IPB Dramaga, Bogor, West Java, Indonesia - 16880; email: hera_maheshwari@yahoo.com

•I Wayan Teguh Wibawan, Department of Animal Disease and Veterinary Public Health, Veterinary Medicine Faculty, Bogor Agricultural University, Jalan Agatis, Kampus IPB Dramaga, Bogor, West Java, Indonesia - 16880, email: teguhwibawan@yahoo.co.id

•Etih Sudarnika, Department of Animal Disease and Veterinary Public Health, Veterinary Medicine Faculty, Bogor Agricultural University, Jalan Agatis, Kampus IPB Dramaga, Bogor, West Java, Indonesia - 16880; email: etih.sudarnika@gmail.com

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