
Analysis of Antibacterial Activity of Antibiotics to Anaerobes of Female Genital Tract in Vitro

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Abstract: This study to investigate predominant anaerobic bacteria and their sensitivity spectrum to antibiotics in female patients with genital tract infections from the local and thus to provide scientific basis for the appropriate use of antibiotics. Anaerobic culture method was used to culture and isolate anaerobes in female patients with genital tract infections. Anaerobes were identified by using micro-biochemistry reacting technique and antibiotic-disc susceptibility test. Antibacterial activity of antibiotics to anaerobes was analyzed by means of Kirby-Bauer testing and broth micro-dilution method. The experiment results showed that 72 strains of obligate anaerobes were isolated from 103 samples, positive rate of 69.90%. The predominant anaerobic bacterial were Gram-negative non-spore bacteroides (28 strains, 38.89%) and anaerobic peptostreptococcus (18 strains, 25%). Sensitivity rate of anaerobic cocci, bacteroides and veillonella to metronidazole was 90.91%, 85.71% and 80% respectively. The drug resistance rates of the three isolated anaerobes were all higher than 60% to amikacin, erythromycin and clindamycin. Comparing the MIC₅₀ and MIC₉₀ values of metronidazole (MTZ), penicillin G (PCG) and lincomycin (LCM) on the isolated anaerobes, MTZ had the lowest MIC₅₀ and MIC₉₀ value. The study suggested that anaerobic infection has become a major pathogenic bacterium of infectious diseases in obstetrics and gynecology. Although MTZ has shown strong antibacterial activity in vitro, a few drug resistant strains appeared in the clinically isolated anaerobes and there is an upward trend of the MIC values of MTZ at different rates.

Keywords: Antibiotics, Anaerobic Infection, Female Genital Tract, Antibacterial Activity

1. Introduction

Anaerobes are part of normal flora parasites to female genital tract. Normal flora has an important role in the part of defense mechanism such as to decompose sugar, to produce acid and to inhibit dwelling of pathogens and so on. Once dysbacteriosis it can lead to infection diseases in female genital tract [1-3]. There has been a lack of appreciation of the significant role anaerobes play in obstetric and gynecologic infections. Utilization of newer appropriate anaerobic culture techniques has shown the importance of these organisms as pathogens in severe infections of the genital tract [4]. Clinically, anaerobes have very high proportion in all infection cases of female genital tract and their infection rate

reaches as high as 73.89% [5]. It was reported that anaerobes were isolated in the secretions of patients with pelvic abscess, appendix abscess, non-venereal Bartholin gland abscess, postpartum endometritis, infection abortion and infection of vaginal residual after hysterectomy. Their positive rates were 80-90%, 63%, 66-70%, 70-93%, 81-100% and 76-80%, respectively [4, 6-8]. Streptococcus anaerobes and bacteroides fragilis are frequently isolated from puerperal infection and infectious abortion. These two anaerobes are easy to breed in endometrial fragments to necrosis of postpartum or residual placental tissues of the patients, which could lead to septicemia when severe infections happen. When lying-in women have septicemia from bacteroides fragilis it could often involve fetus and thus increase the death rate of fetus. If

infection abortion is complicated with uterus gangrene due to *Clostridium perfringens*, the patients could show very serious poisoning symptom, and patients' death rate could be as high as 50% [9]. Anaerobes infection is also related to bacterial vaginosis and nonspecific vaginitis [10, 11]. Therefore, gynecologists and obstetricians must pay a great attention to diagnosis and treatment of anaerobic infections in female genital tract.

At present use of antibiotics is still the major treatment method for anaerobic infections. However, anti-anaerobic drugs and non-antianaerobic drugs are quite different. As the widespread use of antibiotics in recent years, resistance of anaerobes to the drugs is also increasing. It is believed that the resistance rate of anaerobes to metronidazole (MTZ), an effective antibiotic to anaerobes, is about 5 - 10% [12]. There were few reports in China about predominant bacteria of anaerobic infection of female genital tract and the predominant bacteria of anaerobic infection and drug sensitivity spectrum are different for different regions and hospitals. Therefore drug usage would be empirical, based on the drug sensitivity spectrum of the region and laboratory. This study is to analyze predominant anaerobic bacteria and antibacterial activity of antibiotics in local female patients with genital tract infections and to provide scientific basis for the appropriate antibiotics use to treat the anaerobic infections.

2. Material and Method

2.1. General Information

There are 103 cases of untreated acute and chronic genital tract infection cases in the obstetrics and gynecology department of a local hospital. Among them are 35 cases of intrauterine infection, 26 of pelvic abscess, 8 of salpinx abscess, 15 of salpingitis, 11 of postoperative infection, and 8 of puerperal infection. Ages of patients: 22 – 45 years old.

2.2. Reagents

Medium and additive: Nutrient agar (Beijing AoBox Biotechnology Ltd.), CDC Anaerobic Agar, Brucella Broth, Hemin, Vitamin K1 (Qingdao Haibo Bio-technology Co. Ltd.)

Antibiotics: Metronidazole (MTZ, Sichuan Kelun Pharmaceutical Co., Ltd.), Penicillin G (PCG, Northern China Pharmaceutical Co., Ltd.), Lincomycin (LCM, Changzhou Lanling Pharmaceutical Co., Ltd.)

Susceptibility paper: Metronidazole and 8 others (Hangzhou Microbial Reagent Co. Ltd.)

Chemicals: Sodium thioglycolate (Qingdao Rishui Biotechnology Co. Ltd.), anaerobic gas agent (Mitsubishi Gas Chemical Company, Inc.), anaerobic indicator (custom made)

2.3. Equipment

Water insulation thermostatic incubator (Shanghai, PYY-DHS-40×50), Optical Microscope (Japan, Olympus), Anaerobic tank (Mitsubishi Gas, Model C-31), Level II biological safety cabinet (Singapore, Model AC2-451),

U-bottom 8x12 well Polystyrene microplates (Corning Incorporated, USA)

2.4. Sample Collection

Secretions were collected by doctors in the obstetrics and gynecology department according to the method in reference [13]. For Patients with pelvic abscess, Pus was taken from the uterus rectum depression after being disinfected. Uterine secretions were taken with sterilized catheter. The catheter, covered with a protected film pouch, was inserted into uterus and the film pouch was broken to extract the secretions. The collected secretions were placed in sterilized culture-transporting vials containing sodium thioglycolate and immediately sent for testing.

2.5. Isolation, Culturing and Identification of Anaerobes

After collection, samples were immediately inoculated in CDC anaerobic blood agar plates (containing 5% defibrinated sheep blood, 0.01% vitamin K1 and hemin). They were quickly placed in anaerobic tanks with anaerobe-producing gas agent and indicating agent and cultured at 37°C for 48 -72 hours. Suspicious bacteria strains were selected and the Gram stain was performed. This was followed by aero-tolerant test. Suspicious strains were inoculated in blood nutrient agar plate and CDC anaerobic blood agar plate, respectively. The former was placed in aerobic environment and the latter in an anaerobic tank. Both were cultured at 37°C for 48 -72 hours. Based on the growth of bacteria in the two blood plates, obligate anaerobes and facultative anaerobes were identified. The obligate anaerobes were selected and further tested for anaerobes identification in reference to literature [14], including drawing test, capsuling, spore, flagella test, and antibiotics paper test, etc. Biochemical experiments were then performed on the anaerobes using micro biochemical reaction method [15] to identify the strains of anaerobes.

2.6. Sensitivity of Isolated Clinical Anaerobic Strains to Antibiotics

Sensitivity of isolated clinical anaerobic strains to 9 antibiotics was determined by the K-B method according to Literature [16] and drug-resistant strains were selected.

Using U-bottom 8X12 well Polystyrene microplates, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of anaerobes to metronidazole (MTZ), penicillin G (PCG), and Lincomycin (LCM) were determined to refer NCCLS Broth Microdilution Method [14, 17] and with *Bacteroides fragilis* ATCC25295 as quality control strain. The procedure is described as follows.

MTZ, PCG and LCM were dissolved and diluted to 2,560µg/ml (storage solution) with warm distilled water, phosphate buffered saline (PBS) of PH6.0 and PH7.8, respectively. They were stored at -20°C. When in use, the storage solution was diluted with Brucella broth 10 times into application solution and the application solution was further double diluted until the 11th tube so that the antibiotics concentrations in the tubes are 256, 128, 64, 32, 16, 8, 4, 2, 1,

0.5, and 0.25µg/ml.

Preparation of bacterial solution: Defrost the frozen anaerobes and inoculate it in CDC anaerobic blood agar plate. Cultivate in anaerobic environment for 48 hours. Select 3-4 medium size strains, and transplant them into 3ml sodium thioglycolate broth for enrichment. After 24-hour anaerobic culture, the bacterial solutions were diluted to 10^5 - 10^6 CFU/ml for future use.

Method of Operation: On the 96 well microplates, inject 50µl of double-diluted series of antibiotic solutions and 50µl diluted bacterial solutions to well 1 -11 on each row. As a reference the 12th well was added broth without antibiotic solution and 50µl diluted bacterial solutions. Therefore the final antibiotics concentrations are one half of the injected solutions. Shake the plate on a micro-oscillator to mix the solution inside the wells. Cultivate anaerobically for 24-48 hours and make observations.

Analysis of Results: lowest concentration of the well with no bacteria growth would be the MIC. Inoculate the cultures with no turbid growth were inoculated to CDC anaerobic blood agar plate and cultivate for 24 hours, the lowest concentration of the well with no bacteria growth would be the MBC.

3. Results

3.1. Isolation, Culturing and Identification of Anaerobes

Out of 103 clinical samples, isolated and cultivated are 72 obligate anaerobes (69.90%), 23 facultative anaerobes (22.33%), and 8 culture negatives (7.77%). Among them the predominant anaerobic bacteria is anaerobic non-spore forming Gram-negative bacteroides (28 strains, 38.89%), next is peptostreptococcus (18 strains, 25%). (Table 1)

Table 1. Category of Anaerobes from Female Genital Tract Infection.

G staining and morphology	Bacteria Type	Strains	Number of Strains	Subtotal & Percentage
G ⁺ cocci	Peptostreptococcus	P. tetradius	3	18(25%)
		P. prevotii	3	
		P. magnus	4	
		P. productus	2	
		P. anaerobius	6	
G ⁻ cocci	Peptococcus	P. niger	3	10(13.88%)
	Veillonella	V. parvala	8	
		V. alkaligenous	2	
Non-spore forming G ⁻ bacillus	Bacteroides	B. fragilis	16	28(38.89%)
		B. vulgatus	2	
		B. thetaiotamicron	3	
		B. intermedia	2	
		B. asaccharolyticus	2	
		B. melaninogenius	3	
Non-spore forming G ⁺ bacillus	Eubacterium	E. contortum	3	7(9.72%)
		E. lentum	4	
Spore G ⁺ bacillus	Lactobacillus	L. lactobacillus	3	3(4.17%)
	Clostridium	C. perfringens	3	3(4.17%)
Total				72(100%)

3.2. Antibacterial Activity of Antibiotics to the Isolated Clinical Anaerobes

3.2.1. Sensitivity of Anaerobes to 9 Antibiotics

Our results showed that drug resistance rates of peptostreptococcus to rifampicin, chloramycetin, vancomycin were all 27.27%; and the resistance rates were 45.45% and 18.18% to penicillin and piperacillin, respectively. Resistance rates of veillonella to chloramycetin and piperacillin are 30% and 50%, respectively, while the rates to rifampicin, penicillin, vancomycin are all greater than 70%. Resistance rates of bacteroides to rifampicin, chloramycetin are 52.38% and 42.86%, respectively, rates to piperacillin, penicillin, vancomycin are all greater than 60%. Resistance rate of the three anaerobes to amikacin, erythromycin, clindamycin are

also greater than 60%. (Table 2)

3.2.2. MIC and MBC of MTZ, PCG and LCM to Anaerobes

MIC₅₀ (Minimum concentration of half inhibitory bacterium) and MBC₅₀ (Minimum concentration of half bactericidal) of MTZ, PCG and LCM to anaerobes are compared in the following. It is found that to anaerococcus MTZ has lowest MIC₅₀ (2.0µg/ml), which was 4 times lower than that for PCG (8.0µg/ml) and LCM (8.0µg/ml), respectively. To bacteroides the MIC₅₀ of MTZ was 2.0µg/ml, 4 times and 8 times lower than PCG and LCM, respectively. (Table 3) The MBC₅₀ value of MTZ to anaerococcus was 2.0µg/ml, 4 and 8 times lower than that of PCG and LCM, respectively. To bacteroides the MBC₅₀ value is also 2.0µg/ml, 8 times lower than PCG and LCM. (Table4)

Table 2. Sensitivity of Anaerobes to 9 Antibiotics.

Antibiotics	Bacteria Strains									
	Peptococcus (3)		Peptostreptococcus (11)		Veillonella (10)		Bacteroides (21)		Eubacterium (5)	
	S	R	S	R	S	R	S	R	S	R
AK	1	2(66.67)	4	7 (63.64)	3	7 (70)	6	15(71.43)	4	1 (20)
EM	2	1(33.33)	3	8 (72.73)	3	7 (70)	8	13 (61.90)	3	2 (40)
MTZ	3	0 (0)	10	1 (9.09)	8	2 (20)	18	3 (14.29)	5	0 (0)
DA	1	2 (66.67)	3	8 (72.73)	1	9 (90)	5	16 (76.19)	3	2 (40)
RD	2	1 (33.33)	8	3 (27.27)	2	8 (80)	10	11 (52.38)	4	1 (20)
CM	3	0 (0)	8	3 (27.27)	7	3 (30)	12	9 (42.86)	5	0 (0)
PIP	2	1 (33.33)	9	2 (18.18)	5	5 (50)	8	13 (61.90)	2	3 (60)
PCG	2	1 (33.33)	6	5 (45.45)	0	10 (100)	7	14 (66.67)	1	4 (80)
VA	2	1 (33.33)	8	3 (27.27)	0	10 (100)	2	19 (90.48)	1	4 (80)

Note: (1) S – Sensitivity; R – drug resistance

(2) AK-Amikacin; EM-Erythromycin; MTZ -Metronidazole; DA -Clindamycin; RD -Rifampicin; CM -Chloromycetin; PIP -Piperacillin; PCG -Penicillin; VA-Vancomycin

(3) Among 21 bacteroides strains, there are 16 Strains of bacteroides fragilis, 3 strains of bacteroides melaninogenicus and 2 strains of bacteroides vulgatus.

Table 3. Minimum Inhibitory Concentration (MIC) of Antibiotics to Anaerobes.

Antibiotics	Anaerobes strains	Minimum Inhibitory Concentration ($\mu\text{g/ml}$)											MIC ₅₀	MIC ₉₀
		≤ 0.125	0.25	0.50	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128		
MTZ (1)	Anaerococcus (24)	3	0	3	3	4	5	2	2	2	0	0	2.0	16.0
	Bacteroides (20)	2	3	0	4	2	6	1	2	0	0	0	2.0	8.0
	Eubacterium (3)	0	0	2	1	0	0	0	0	0	0	0	-	-
PCG (2)	Anaerococcus (22)	0	0	1	2	0	4	6	5	2	1	1	8.0	32.0
	Bacteroides (21)	0	0	2	0	2	3	4	2	5	2	1	8.0	32.0
	Eubacterium (3)	0	0	0	0	0	0	0	2	1	0	0	-	-
LCM (3)	Anaerococcus (20)	0	0	2	0	2	1	4	3	3	2	3	8.0	64.0
	Bacteroides (21)	0	1	1	0	0	1	2	5	2	6	4	16.0	64.0
	Eubacterium (3)	0	0	0	0	0	1	2	0	0	0	0	-	-

Note: (1)24 anaerococcus include: 2 peptococcus, 14 peptostreptococcus, 8 veillonella, 20 bacteroides include 15 bacteroides fragilis, 2 bacteroides vulgatus and 3 bacteroides melaninogenicus

(2) 22 anaerococcus include: 2 peptococcus, 12 peptostreptococcus, 8 veillonella, 21 bacteroides include 15 bacteroides fragilis, 3 bacteroides thetaiotaomicron and 3 bacteroides melaninogenicus

(3) 20 anaerococcus include: 2 peptococcus, 12 peptostreptococcus, 6 veillonella, Categories of 21 bacteroides were the same 2

2. MIC₅₀₍₉₀₎: Minimum concentration of half (90%) inhibitory bacterium

MBC₅₀₍₉₀₎: Minimum concentration of half (90%) bactericidal

Table 4. Minimum Bactericidal Concentration of Antibiotics to Anaerobes ($\mu\text{g/ml}$).

Antibiotics	Anaerobes strains	MBC range	MBC ₅₀	MBC ₉₀
MTZ	Anaerococcus (24)	$\leq 0.125 \sim 64$	2.0	16.0
	Bacteroides (20)	$\leq 0.125 \sim 32$	2.0	16.0
PCG	Anaerococcus (22)	$1.0 \sim \geq 128$	8.0	64.0
	Bacteroides (21)	$1.0 \sim \geq 128$	16.0	64.0
LCM	Anaerococcus (20)	$1.0 \sim \geq 128$	16.0	64.0
	Bacteroides (21)	$0.25 \sim \geq 128$	16.0	64.0

4. Discussion

Anaerobic bacteria are opportunistic pathogens. Under normal circumstances, they are parasitic to production channel and may not lead to sickness. However, when body's defenses is weak or being weakened due to injury, surgery, giving birth, local blood deficiency, damaged or necrotic tissue, foreign objects such as IUDs, exogenous microorganisms grow and reproduce to cause tissue damage. Bacteria in the vagina could

migrate up to intrauterine and oviduct. If accompanied with aerobic bacteria, it provides a good environment for the anaerobes to grow and reproduce, causing genital inflammation [18]. After the infection, inappropriate use of antibiotics could lead to dysbacteriosis and the increase of anaerobic infections [19]. In this study 72 strains of obligate anaerobes were isolated from 103 clinical samples of female reproductive tract infections and the positive rate is 69.9%, close to the rate of 68% reported by Zhang *et al.* [20]. Among the 72 anaerobes there are 18 peptostreptococcus (25%) and 16 bacteroides fragilis (22.22%), different from those obtained by Chen *et al.* (30.15% and 12.69%) [21]. There are also 8 strains of veillonella (11.11%), which is higher than the results from Zhang (1.2%) and Zhao (7.4%) [20, 22].

Both cephalosporin and penicillin have β -lactam ring in their molecular structures. Their bacteria-fighting mechanism is to suppress the formation of bacteria cell wall and destroy the bacteria's physiological functions, thus avoiding the infection. However, as the antibiotics are widely used in recent

years, there is a gradual increase in the β -lactamase species and active spectra. Abuses of β -lactam antibiotics further increase number of bacteria that contain β -lactamase, making the drug less effective. Fragile bacteroides are the most common opportunistic pathogens in clinical non-spore forming anaerobes. Its drug resistance mechanisms for β -lactam ring antibiotics are: (1) most bacteroides fragilis can produce β -lactamase [23] and break up the β -lactam ring, making it inactive; (2) β -lactamase can quickly combine with β -lactam drugs so that the drugs remain in between the cell walls and cannot reach and work with penicillin, making it drug resistance [24]. In this study we analyzed the sensitivity of several isolated clinical anaerobes to MTZ and other antibiotics. Our results show that the drug resistance rates of bacteroides (mainly fragile bacteroides) to penicillin and clindamycin were 66.67% and 76.19%, respectively. The former were lower than those reported by Cao and Fan's results (100%、78.6%) and the latter were higher than those reported by Cao and Fan's results (20%、28.6%) [25, 26]. The rates for piperacillin and chloromycetin were 61.90% and 42.86% respectively, both of which were higher than the results by Cao et al [25]. Resistant rate of bactericides to vancomycin, erythromycin were 90.48%, 61.90% respectively. The results were higher than those reported by Zhou et al (45.10%, 46.13%) [27]. Sensitivity rates of peptostreptococcus, bacteroides and veillonella to MTZ are the highest, being 90.91%, 85.71% and 80% respectively. Besides MTZ, other antibiotics that peptostreptococcus is highly sensitive to are piperacillin, rifampicin, chloromycetin, and vancomycin. Among them the rate is 81.82% for piperacillin and 72.73% for the other three. The sensitive rates for MTZ and piperacillin in vitro were in agreement with the treatment results of anaerobic infections in female genital tract with these two drugs reported by Creasas and Sweet et al. [28, 29]. The antibiotics that veillonella is highly sensitive to is chloromycetin (70%), but the rate was lower than those reported by wang et al (100%) [30].

After analyzing the antibacterial activity of MTZ, PCG and LCM to various anaerobes, we found that MIC to suppress 50% and 90% bacteroides were 2.0 μ g/ml and 8.0 μ g/ml for MTZ and they are higher than those obtained by zhang et al (0.25 μ g/ml, 4.0 μ g/ml), respectively [31]. MIC to suppress 50% and 90% anaerococcus were 2.0 μ g/ml and 16.0 μ g/ml respectively, and the former was lower than Zhou's results and the latter was same as those reported by Zhou et al. (8.0 μ g/ml, 16.0 μ g/ml) [32]. MIC₅₀ value of LCM to anaerococcus and bacteroides were 8.0 and 16.0 μ g/ml respectively, and they were higher than Wang's results (2.0 μ g/ml, 2.0 μ g/ml). MIC₉₀ values of LCM to the two anaerobes were both 64.0 μ g/ml. The results are higher than those reported by Wang et al (16.0, 16.0 μ g/ml), respectively [33]. MIC₅₀ values of PCG to the two anaerobes were both 8.0 μ g/ml and MIC₉₀ values were both 32.0 μ g/ml. The former were half of Deng's results (16.0 μ g/ml), the latter were the same as Deng's results [34]. Our results shows that among the three MTZ has the strongest antibacterial activity and is still the drug of first choice for anaerobic infections.

5. Conclusion

In this study 72 anaerobes were isolated from samples taken from 103 female reproduction tract infection patients. Our analysis demonstrates that anaerobic bacteria and gynecological infectious diseases are closely related. Anaerobic infection has become an issue in clinical gynecology that cannot be ignored. Although our in vitro experiments showed high sensitivity of isolated clinical anaerobes to MTZ, some strains appear drug-resistant. For this group of data, drug resistance rate of bacteroides to MTZ is 14.28%, higher than 11 years ago (5–10%) [12]. MIC₅₀ and MIC₉₀ values of MTZ to bacteroides are also significantly higher than 15 years ago [31]. This indicates that the resistance of anaerobic bacteria to MTZ is gradually increasing and should cause attention of clinical workers.

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