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Occurrence of Metronidazole-Resistant *Trichomonas vaginalis* in Japan

QI HONG YE[1,2], SEIKI KOBAYASHI[1], TATSUSHI FUJIWARA[1], SHIRO NOZAWA[3], HIROAKI OHTA[3], KAZUNARI KIGUCHI[3], RYUTARO TOJO[4], JUN-ICHI KOBAYASHI[5], KI-ICHIRO SUMI[6], AKIHIKO YANO[7] AND TSUTOMU TAKEUCHI[1]

[1]*Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160, Japan.*
[2]*ShenYang Anti-Epidemic and Sanitary Station, Huang Gu District, ShengYang, China.*
[3]*Department of Obstetrics and Gynecology, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160, Japan.*
[4]*Tojo Women's Clinic, Konan-ku, Yokohama 233, Japan.*
[5]*Division of Obstetrics and Gynecology, Saiseikai Kanagawa Hospital, Kanagawa-ku, Yokohama 221, Japan.*
[6]*Division of Obstetrics and Gynecology, Nippon Steel Hospital, Kawasaki-ku, Kawasaki 210, Japan.*
[7]*Department of Medical Zoology, School of Medicine, Nagasaki University, Nagasaki 852, Japan.*
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Abstract

Susceptibility of *Trichomonas vaginalis* isolates to metronidazole was evaluated *in vitro* under aerobic and anaerobic culture conditions. Reliability of a commercial medium "Fuji" for this purpose was confirmed by evaluating the growth and susceptibility to metronidazole of a reference strain of the protozoon, TS-1:KEIO. Seventeen isolates of *T. vaginalis* were obtained from 5 hospitals in Tokyo, Kanagawa and Nagasaki. In contrast to 16 of the 17 isolates as well as TS-1 strain, virtually all of which had minimum lethal concentration (MLC) of metronidazole of less than 7.5 μg/ml under the anaerobic assay condition, one of the isolates, KO-12 strain, survived at 7.5 μg/ml of metronidazole, the highest concentration tested in this study, and showed a significant growth from the 10th day of cultivation. This strain had been isolated from a patient with recurrent vaginal trichomoniasis treated in two courses of 500 mg oral metronidazole twice daily for 7 days with vaginal suppository of the same drug at 250 mg/day for 7 days. Under the aerobic assay condition, this strain showed MLC of 30 μg/ml, while that of KO-22 strain isolated from the same patient after the 2nd course of treatment was 60 μg/ml. Electron microscopic observation on KO-12 strain with or without 5 μg/ml metronidazole showed ultrastructural changes in hydrogenosome like aggregation of the organelle in the presence of metronidazole. These findings suggest that KO-12 strain is the first isolate of metronidazole-resistant *T. vaginalis* under the anaerobic condition in Japan. A definitive conclusion could not be drawn concerning the aerobic resistance of this protozoon because of the difference in the assay methods employed.

Key words: *Trichomonas vaginalis*; protozoa, parasitic; metronidazole; drug resistance.

Introduction

Metronidazole, a 5-nitroimidazole derivative widely employed for treatment of some protozoan and bacterial infections, was developed and first applied against vaginal trichomoniasis in France in 1960. Two years later, a case of trichomonal vaginitis which showed resistance to the treatment with this drug was reported (Robinson, 1962). However, McFadzean *et al.* (1969) examined the *in vitro* susceptibility to metronidazole of 25 isolates of *T. vaginalis* from the patients who could not be successfully treated with this drug, and found that all of the isolates had the minimum lethal concentration (MLC) comparable to those of the susceptible strains. Consequently, they concluded that such a failure in treatment was ascribed to the spontaneously low-

Correspondence: Tsutomu Takeuchi
斉　宏業[1,2]，小林正規[1]，藤原達司[1]，野沢志朗[3]，太田博明[3]，木口一成[3]，東条龍太郎[4]，小林淳一[5]，角毅一郎[6]，矢野明彦[7]，竹内　勤[1]([1]慶応義塾大学医学部熱帯医学・寄生虫学教室，[2]瀋陽市衛生防疫站，[3]慶応義塾大学医学部産婦人科学教室，[4]東条ウィメンズクリニック，[5]済生会神奈川病院産婦人科，[6]日本鋼管病院産婦人科，[7]長崎大学医学部医動物学教室)

ered or impaired absorption of metronidazole by bacteria. Later, Meingassner and Thurner (1979) first reported the occurrence of metronidazole-resistant *T. vaginalis* on the basis of *in vitro* and *in vivo* experiments. Subsequent studies (Lossick *et al.*, 1986; Lossick, 1989; Tachezy *et al.*, 1993) showed that there were significant differences in the mechanism and intensity of metronidazole resistance of this protozoon between under aerobic and anaerobic assay conditions.

On the other hand, metronidazole-resistant *T. vaginalis* has not been reported in Japan. Though there have been numerous cases with trichomoniasis, who could not be successfully treated by metronidazole, the fact that even the human infection with the drug resistant *T. vaginalis* can be treatable by increasing the doses twice or thrice as much as the standard prescription (Tachezy *et al.*, 1993) may have induced our insufficient attention to the drug resistance in Japan. Under such circumstances, we once examined the occurrence of metronidazole-resistant *T. vaginalis* (Asami *et al.*, 1972); however, we could not find any of such strains. In the present study, we further tried to evaluate if the susceptibility of *T. vaginalis* has been changing in Japan.

Materials and Methods

Isolation of T. vaginalis

In collaboration with Keio University Hospital, Tojo Womens' Clinic, Saiseikai Kanagawa Hospital, Nippon Steel Hospital and Department of Medical Zoology, School of Medicine, Nagasaki University, *T. vaginalis* was isolated and cultivated as follows from the patients with vaginal trichomoniasis.

Media

Trichomonal culture medium "Fuji" (Fuji Pharmaceutical Co., Toyama, Japan), which was designed on the basis of Diamond's TYM medium (Diamond, 1957), was employed for isolation of trichomonads from the patients' specimens according to the instruction of manufacturer. For establishment of the axenic strains and its maintenance, BI-S-33 medium (Diamond *et al.*, 1978) was employed. To evaluate the drug susceptibility of *T. vaginalis*, the medium "Fuji" was used.

Metronidazole

Metronidazole was supplied by the courtesy of Shionogi Pharmaceutical Co. (Tokyo, Japan) as a purified powder for experimental purposes. It was dissolved in distilled water to make 450 μg/ml and 6 mg/ml for the anaerobic and aerobic experiment, respectively. These stock solutions were sterilized by filtering through a Millipore membrane filter (pore size: 0.22 μm) and maintained at 4°C. Immediately before use, the stock solutions were diluted in distilled water.

Establishment of axenic T. vaginalis

Immediately after the definitive diagnosis of vaginal trichomoniasis was made, vaginal discharge was inoculated into the medium "Fuji" according to the manufacturer's instruction. Because this medium changes its color from red to yellow as *T. vaginalis* grows (Takada *et al.*, 1990), the culture was maintained at 35.5°C for 48 hours and monitored for the change of color. After the color change was recognized, trichomonads were subcultured to the agar-free BI-S-33 medium in a No. 15 screw-capped pyrex culture tube (13×100 mm). Air content of the medium was made minimum by adding the culture fluid to the tube as much as possible to reduce the growth of such aerobic fungi as *Candida* or *Torulopsis*. Contamination with fungi was further prevented by repeated centrifugation of the culture tubes followed by keeping the tubes vertically so that only actively motile trichomonads could be recovered in the supernatant fluid. Growth of the contaminated bacteria was inhibited by antibiotics originally present in the medium; moreover, organic acids produced by *T. vaginalis* in the culture as its metabolic endproducts also could suppress the growth of bacteria. Eventually, by these procedures, virtually all of the cultures were judged free from contaminated bacteria during subculturing twice or thrice as examined light microscopically.

Examination of susceptibility of the isolates to metronidazole

Since the *in vitro* drug susceptibility of *T. vaginalis* is affected by constituents and gas phase of the assay medium, a single lot of the medium "Fuji" was employed throughout. After ampoules containing the medium were opened, it was transferred to the

No. 15 screw-capped pyrex culture tube to make observation of the culture by an inverted light microscope easier.

Assessment of the susceptibility in the anaerobic study was conduced as follows. Five and half ml of the medium "Fuji" was inoculated into each of the pyrex culture tubes. To this medium, 6×10^5 trichomonads, which were harvested, washed on the 2nd day of cultivation in BI-S-33 medium and suspended in 0.5 ml of the medium "Fuji", were added. The test tubes were rotated to make the culture media homogeneous and vertically placed to cultivate at 35.5°C. The growth of *T. vaginalis* was confirmed in 24 hours by counting the number of actively motile trichomonads in 0.1 ml of the culture medium which was made homogeneous. Subsequently, 0.1 ml of the stock solutions of metronidazole was added to yield the final concentration of 2.5, 5.0 and 7.5 μg/ml. This experiment was done in duplicate. As the control, 0.1 ml of distilled sterile water was added. MLC was defined as the lowest concentration of this drug among those tested at which the parasite was completely killed during observation for 2 weeks, which was confirmed by the lack of resumption of the growth for subsequent 2 weeks. The growth of trichomonads was evaluated using average number of the viable protozoon at each concentration of metronidazole, which was counted with a hemocytometer from the 2nd day after adding the drug solution for 2 weeks at the interval of 24 hours. Viability of trichomonads could be easily assessed on the basis of movement of flagella and/or undulating membrane.

Evaluation of the susceptibility under the aerobic condition was done essentially in the same manner as above except for the followings. The amount of culture medium and the number of *T. vaginalis* inoculated was 2.5 ml and 3×10^5 in 0.5 ml of the culture medium, respectively. The gas phase was made as aerobic as possible by loosely fitting the screw cap of the culture tube. In addition, in order to increase the contact area between the culture medium and air, the culture tubes were placed obliquely with the angle of 5~6°C against the horizontal plane. The final concentration of metronidazole was set at 7.5, 15, 30, 60 and 120 μg/ml by adding 0.1 ml of the drug solution. Since preliminary study showed that trichomonads were killed in 2 days after adding 7.5 μg/ml metronidazole, MLC was defined as the lowest concentration of metronidazole needed for significant suppression of the growth during observation for 24 hours after adding the drug.

Electron microscopic observation

Both of the parasite grown in the presence and absence of 5 μg/ml metronidazole were processed for electron microscopy. After the logarithmic growth *T. vaginalis* was confirmed on the 7th day of cultivation following a decay of the trichomonad-cidal effect of metronidazole, both of the cultures were chilled in an ice bath for 10 min to detach the parasite from the culture tubes and centrifuged at 450×g for 5 min to remove the supernatant fluid. The pellet was made as homogeneous as possible and added with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for prefixation for 60 min at 4°C. After washing thrice in 0.1 M cacodylate buffer during 3 hours, the specimens were postfixed by 1% osmium tetroxide in the same buffer for 90 min at 4°C. Then, the specimens were processed in the conventional manner and observed with a Hitachi HU-12AS electron microscope (Hitachi Ltd, Tokyo, Japan), after staining with uranyl acetate and lead citrate.

Results

We could isolate 17 strains of *T. vaginalis*, generally named in the order of isolation, in this study. Anaerobic assessment of the metronidazole resistance was conducted against all of these 17 isolates, whereas the aerobic study was done against 4 strains on the basis of findings by the anaerobic study.

Data on the anaerobic study were summarized in Fig. 1. We could find one isolate, KO-12 strain, which showed significant growth in the presence of 7.5 μg/ml metronidazole during 2 weeks. The other 16 isolates were judged to have MLC of 2.5, 5.0 or 7.5 μg/ml, since they did not resume growth during the period of observation.

For comparing with the characteristics of KO-12 strain, effects of metronidazole on the growth kinetics of KO-6 strain, which was isolated from another case of vaginal trichomoniasis and seemed to have standard MLC, i.e., 5.0 μg/ml, among the strains isolated in the present study, was examined (Fig. 2). The lethal effect of metronidazole was evident at 5.0

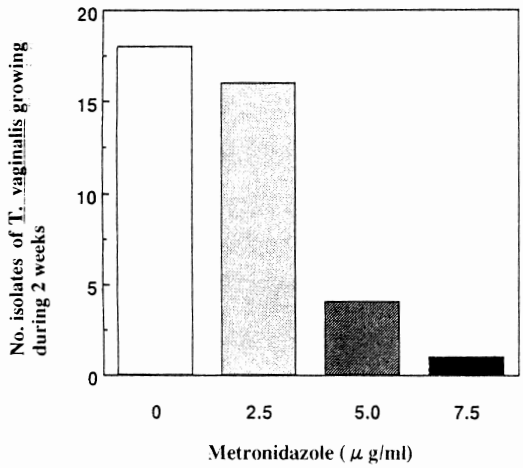

Fig. 1 *In vitro* anaerobic assessment of the susceptibility of *Trichomonas vaginalis* isolates to metronidazole.

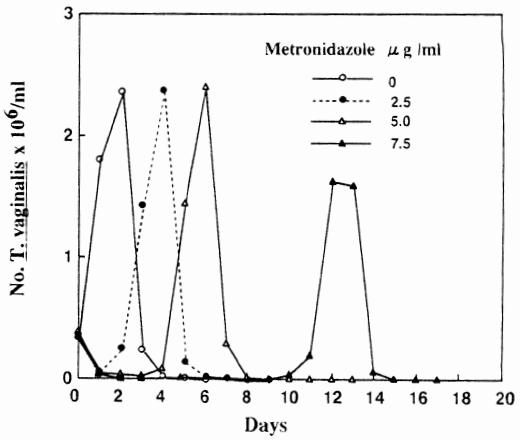

Fig. 3 Growth of a metronidazole-resistant *Trichomonas vaginalis* isolate (strain KO-12) under anaerobic condition.
Metronidazole was added on the day 0.

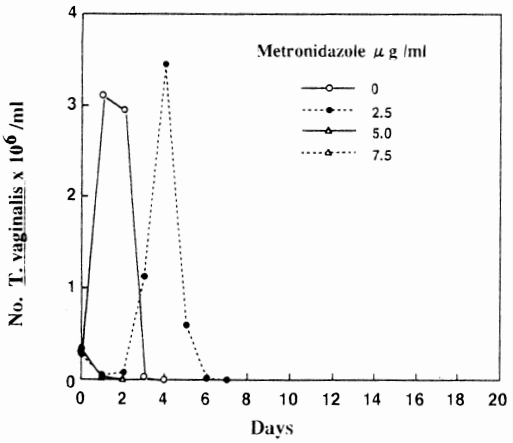

Fig. 2 Growth of a metronidazole-susceptible *Trichomonas vaginalis* isolate (strain KO-6) under anaerobic condition.
Metronidazole was added on the day 0.

μg/ml before the 2nd day of cultivation, while at 2.5 μg/ml, trichomonads rapidly increased in number from the 2nd day. On the other hand, KO-12 strain was found to significantly grow on the 3rd, 5th and 12th day of cultivation in the presence of 2.5, 5.0 and 7.5 μg/ml, respectively (Fig. 3). However, the MLC of KO-22 strain, isolated from the same patient as KO-12 strain on 7th day after completion of the 2nd course of the drug administration despite the negative light microscopic examination, was 5.0 μg/ml. We can not completely rule out the possibility that KO-22 strain was originated from reinfection with another strain of *T. vaginalis* after completion of the 2nd course of chemotherapy; however, it seems quite unlikely that the reinfection occurred during such a short period as 7 days after the 2nd course, as the patient had no partner during the period of observation. The finding that we could isolate *T. vaginalis* from the patient between the 1st and 2nd treatment may also support the view.

In the aerobic study, 4 out of the 17 strains including KO-12 and KO-22 tested in the anaerobic study were examined. Strain TS-1:KEIO, isolated and maintained in our laboratory as a reference strain of *T. vaginalis* on the basis of a series of characterizations, and other 5 strains, also isolated and maintained in the same manner, with the anaerobic MLC of 5 μg/ml (Ye *et al.*, unpublished observation) were tested for comparison. The MLC value of KO-12 strain was unexpectedly low, i.e., 30 μg/ml, while KO-22 strain showed a high MLC, i.e., 60 μg/ml (data not shown). All of the compared strains had the MLC of 15 μg/ml.

Table 1 and 2 summarize the profiles and treatment data of the patient from whom KO-12 and KO-22 strains were isolated. The patient was a 35 years

old Japanese female. Through cytological examination under the diagnosis of adenomyosis uteri, *T. vaginalis* was detected. Chemotherapy against trichomonal infection was started immediately in two courses of the drug administration with intermission of one month. In each course, the dose administered was oral 500 mg metronidazole twice daily together with vaginal suppository of 250 mg metronidazole daily for 7 days.

Table 1 Profile and history of the patient from whom KO-12 and KO-22 strains were isolated

Profile:		
Nationality:	Japanese	
	Female, 35 years old	
	Single at this study	
Occupation:	An employee of a company	
Childbirth:	once at the age of 25	
Artificial abortion: twice at the age of 19 and 27		
Case history:		
1992, October	03:	Endometriosis
1994, April	05:	Adenomyosis uteri (Chocolate cyst)
1996, June	15:	Diagnosed class II on the basis of the criteria of cytodiagnosis of cancer. *Trichomonas vaginalis* was detected on the stained specimen.
1994, June	28:	Recognized abdominal pain after menstruation. Definitively diagnosed as trichomonal vaginitis.

Fig. 4 demonstrates ultrastructure of KO-12 strain grown in the absence of metronidazole for 2 days, whereas Fig. 5 shows the same strain under the logarithmic growth by cultivating for 7 days in the presence of 5 μg/ml metronidazole. These figures clearly demonstrate aggregation of hydrogenosomes, which are usually located regularly along axostyle or costa of trichomonad, and fusion of the hydrogenosomal membrane in the presence of metronidazole.

Discussion

Since difference in the MLC between the susceptible and metronidazole-resistant *T. vaginalis* was found to be more clearly demonstrable under the aerobic assay condition (Meingassner *et al.*, 1978; Meingassner and Thurner, 1979), standardized criteria of the metronidazole resistance have been increasingly definite. For instance, Lossick *et al.* (1986) defined metronidazole-resistant *T. vaginalis* as the protozoon which can grow at more than 50 μg/ml metronidazole under the aerobic condition and the highly resistant strain as that growing at more

Table 2 Process of chemotherapy with metronidazole of the patient from whom KO-12 and KO-22 strains were isolated

Date	Metronidazole administration	Detection of *T. vaginalis*	
		Microscopic examination	Cultivation
1994.06.28	500 mg* twice daily × 7 days 250 mg† once daily × 7 days	+	+ (KO-12)
1994.07.07	not done	–	not done
1994.08.23	500 mg* twice daily × 7 days 250 mg† once daily × 7 days	+	+
1994.09.05	not done	–	+ (KO-22)

*Oral administration
†Vaginal suppository
Microscopic examination was done with a vaginal discharge.
Cultivation was done utilizing the medium "Fuji". Other details as in the text.

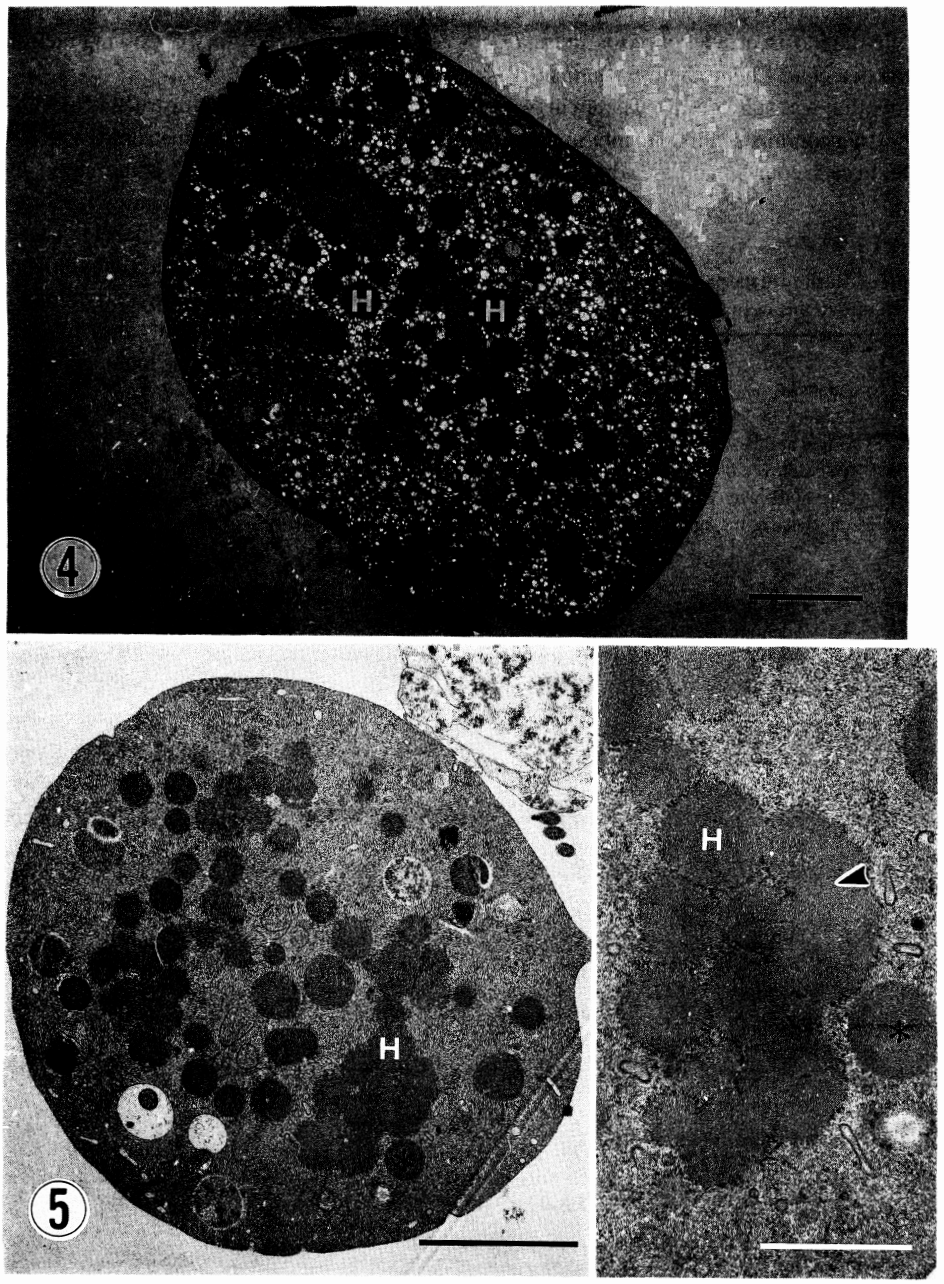

Fig. 4 An electron microscopic view of KO-12 strain of *Trichomonas vaginalis* grown in BI-S-33 medium without metronidazole for 2 days. Each of the hydrogenosomes (H) were arranged separately. Bar = 2.5 μm

Fig. 5 KO-12 strain grown for 7 days in the same medium containing 5 μg/ml metronidazole. The hydrogenosomes (H) were arranged irregularly, often aggregated or fused each other (black arrow head) as compared with Fig. 4. The highly magnified picture shows fusion of the hydrogenosomal membrane. Black bar = 2.5 μm. White bar = 1 μm. *the magnified portion.

than 201 μg/ml metronidazole. They also postulated that the successful treatment of such a drug resistant *T. vaginalis* infection requires oral administration of 3.0 to 3.5 g daily for 14 days and the simultaneous vaginal suppository of 500 to 1,000 mg of metronidazole. On the other hand, concerning the anaerobic resistance, Kulda *et al.* (1993) demonstrated that such a resistance as MLC of 10 μg/ml could be maintained as a stable property of this protozoon, which suggests that this is the common level of the anaerobic resistance among the clinical isolates. Müller (1983) showed that a rate in the treatment failure increased if *T. vaginalis* isolates had the anaerobic MLC >1.8 mg/l. On the basis of these data, Lossick (1989) suggested that the normal anaerobic metronidazole susceptibility for *T. vaginalis* was less than 3.0 mg/l.

Concerning the difference in MLC between aerobic and anaerobic conditions, Tachezy *et al.* (1993) suggested that the MLC of strain MRP-2MT, which seemed to be a standard metronidazole-resistant strain (Kulda *et al.*, 1982), was 88.2 μg/ml and 6.3 μg/ml under the aerobic and anaerobic condition, respectively. Moreover, among experimentally induced metronidazole resistant strains of *T. vaginalis*, the most resistant one, which showed MLC at 261.5 μg/ml by the aerobic analysis, indicated the anaerobic MLC of 4.2 μg/ml (Kulda *et al.*, 1982). These previous studies strongly suggest that the aerobic resistance is not necessarily parallel to the anaerobic resistance. Consequently, Kulda *et al.* (1982) considered that the aerobic and anaerobic metronidazole resistance of *T. vaginalis* were independent phenomena. Indeed, even by experimental manipulation, it has been difficult to induce highly resistant *T. vaginalis* to metronidazole under the anaerobic condition; however, the aerobically resistant protozoon could be easily established (Tachezy *et al.*, 1993).

In our present study, metronidazole susceptibility was evaluated from the growth or sterilizing velocity of *T. vaginalis*; accordingly, there might be minor discrepancies between our data and those of Meingassner *et al.* (1978) who first reported the method of evaluation of metronidazole resistance of this protozoon. However, KO-12 strain showed essentially the same susceptibility to metronidazole as examined anaerobically according to the original procedure of Meingassner *et al.* (1978) (Ye *et al.*, unpublished observation). Moreover, this strain could resume the growth in the presence of 7.5 μg/ml. These findings suggest that KO-12 strain is included in the entity of anaerobically resistant *T. vaginalis* to metronidazole, though it is not resistant under the aerobic condition. On the other hand, it seems inappropriate to draw a definite conclusion on the aerobic resistance of KO-22 strain, since there is a significant difference in the assay method between us and Meingassner *et al.* (1978), who determined MLC on the basis of the number of viable trichomonads in 2 days after drug addition. However, these studies led us to conceive that KO-22 strain was aerobically much less susceptible to metronidazole than any other strains tested in this study.

Recently, the prevalence of trichomonal infection has been decreasing in Japan. According to the statistics by Japanese Ministry of Health and Welfare, the prevalence decreased from 100 in 1987 to 60.5 in 1990 (Infectious Diseases Surveillance Information, 1992). Takada (1991) also reported that the rate of detection of *T. vaginalis* had been decreasing. They detected this protozoon at 33% from females with vaginal discharge in 1972, whereas the rate was 20% in 1991. Takada (1991) considered that extensive use of oral 5-nitroimidazoles to treat vaginal trichomoniasis without constant care might be at least partially responsible for such a decrease in the prevalence. It seems possible that such an extensive use of oral metronidazole might also increase the resistance of *T. vaginalis* to this drug. Our present study clarified the occurrence of *T. vaginalis* which was judged to be resistant to metronidazole under the anaerobic condition in Japan and also prompts us to pay more attention to the metronidazole resistance.

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