CROSS-RESISTANCE RELATIONSHIPS BETWEEN PAROMOMYCIN (AMINOSIDINE), LIVIDOMYCIN, KANAMYCIN, AND CAPREOMYCIN RESISTANCES OF *MYCOBACTERIUM TUBERCULOSIS*

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Introduction

Paromomycin[1~4] and aminosidine[5] are aminoglucoside antibiotics recently discovered. Identity of paromomycin and aminosidine has been reported[6]. Knowledge of cross-resistance-relationships in respect to this antibiotic is very scanty. ARCAMONE *et al.*[5] stated that a neomycin-resistant strain of *Staphylococcus aureus* was resistant to paromomycin.

The present study was carried out in an aid to know cross-resistance-relationships of *Mycobacterium tuberculosis* in respect to this antibiotic and other aminoglucoside antibiotics and capreomycin.

Methods

Mycobacterium tuberculosis, $H_{37}Rv$ and Aoyama-B and eleven strains freshly isolated from patients, were used. The latter eleven strains were isolated from tuberculous patients previously not treated with any antituberculous agent by single colony-isolation technique.

Tests for the resistance to various antituberculous agents were carried out by the "actual count" method[7~9], and the degree of resistance was expressed as the highest concentration of an agent on which a small inoculum consisting of 20 to 100 viable units could form visible colonies after incubation at 37°C for 4 weeks. OGAWA egg medium was used throughout. The composition of the OGAWA egg medium is as follows: Basic solution (1% KH_2PO_4 and 1% sodium glutamate), 100 mL; whole eggs, 200 ml; glycerol, 6 ml; 2% malachite green solution, 6 ml (resulting pH 6.8). The medium was poured at 8 ml quantities into tubes, 17 by 170 mm, and made as slopes by sterilization at 95°C for 60 minutes.

Antituberculous agents were added to the medium before the sterilization. The activity of agents was expressed as a base value. The following abbreviations are used in tables and figures: Dihydrostreptomycin sulfate=SM; isoniazid=INH; sodium p-aminosalicylate=PAS; ethionamide=TH; ethambutol=EB; rifampicin=RFP; kanamycin sulfate=KM; capreomycin sulfate=CPM; lividomycin sulfate=LVM; paromomycin sulfate=PM; viomycin sulfate=VM.

The present study mainly concerns with cross-resistance-relationships between kanamycin, capreomycin, lividomycin and paromomycin resistances. The manufacturers of these agents are the Meiji Seika Company, Tokyo, the Eli Lilly and Company, Basingstoke (England), the Kowa Company, Nagoya, and Kyowa Fermentation Company, Tokyo, respectively.

The test strain growing on OGAWA egg medium was homogenized by shaking with glass beads and made as a suspension by addition of saline (0.9% NaCl solution). From this suspension, a series of ten-fold dilutions were prepared. A 0.02 ml sample of each dilution was inoculated with a spiral loop to a series of OGAWA egg medium containing various concentrations of agents. Control medium without agent was included to the series. The media were incubated at 37°C for 4 weeks. The degree of resistance was read, in a series where control medium showed 20 to 100 colonies, as the highest concentration of agent, in which the small inoculum could form colonies.

Resistant mutant strains were isolated as follows. A single colony was picked up from a medium on which colonies grew as discrete colonies. The colony was named and cultivated on OGAWA egg medium containing no agent. Strain name, for example, PM 1,000 R means a strain isolated on a medium containing 1,000 μg/ml paromomycin. If the colony was still small at 4 weeks, it was picked up at 6 weeks.

To simplify the expression of data, growth of any colony on a drug-containing medium was expressed simply as (+), and absence of growth as (−). Here, the term "growth" indicates growth

of colonies on a drug-containing medium inoculated with a small inoculum consisting of 20 to 100 viable units. The number of viable units inoculated to each medium is counted on control medium, which is inoculated with the same inoculation size.

Results and Discussion

Resistance pattern of *M. tuberculosis* $H_{37}Rv$ to paromomycin.

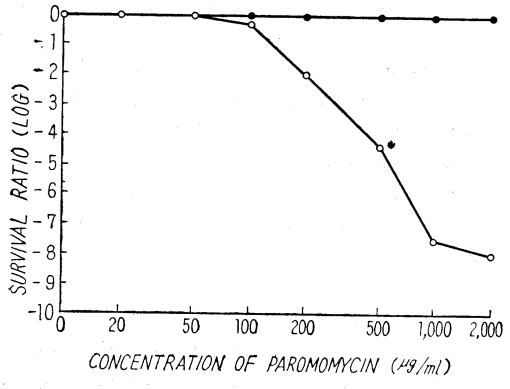
Mycobacterium tuberculosis $H_{37}Rv$ previously not exposed to any agent showed resistance to 100 μg/ml paromomycin and remained susceptible to 200 μg/ml (Fig. 1). KAWAMORI *et al.*[10] stated that the minimal inhibitory concentration of paromomycin for *M. tuberculosis* $H_{37}Rv$ was 1.25 to 2.5 μg/ml in DUBOS liquid medium and 10 μg/ml in KIRCHNER's semiliquid medium. It is evident that the activity of the antibiotic is reduced markedly in an egg medium.

Single colonies growing on various concentrations were picked up, and strains originated from these colonies were tested for their degree of resistance.

Three strains were isolated from medium containing 100 μg/ml, 200 μg/ml, 500 μg/ml, 1,000 μg/ml, and 2,000 μg/ml, respectively. The strains isolated on medium containing 100 μg/ml to 500 μg/ml (the colonies growing on 500 μg/ml were minute at 4 weeks) showed the same survival curve as showed the parent strain and proved to be susceptible as the parent strain. The strains isolated on medium containing 1,000 μg/ml and 2,000 μg/ml were resistant to 2,000 μg/ml or more. The colonies obtained at concentration of 1,000 μg/ml are considered to be resistant mutants, as these retain heritable character of resistance. The strains were either susceptible to the agent as the parent strain or resistant to a high concentration of agent (Fig. 1), and only one phenotype of resistance was observed. The pattern of paromomycin resistance of this organisms is a "single step-pattern".

Mutation frequency to paromomycin resistance (ratio, number of resistant mutants per total viable population) is about 3×10^{-8} (Fig. 1).

Fig. 1. Pattern of paromomycin resistance in *M. tuberculosis* $H_{37}Rv$.
Survival curve shown by open circles is of the parent strain and of strains (3 strains for each concentration) isolated at concentrations of 100, 200 and 500 μg/ml paromomycin.
Survival curve shown by closed circles is of the strains isolated at concentrations of 1,000 and 2,000 μg/ml paromomycin (3 strains for each concentration).
* Colonies incapable of growing at 4 weeks but capable of growing at 6 weeks.



Cross-resistance-relationships between paromomycin resistance and resistances to other antituberculous agents in *M. tuberculosis* $H_{37}Rv$ and Aoyama-B

Susceptibility to paromomycin of various resistant strains and susceptibilities to various agents of

Table 1. Susceptibility to paromomycin of *M. tuberculosis* $H_{37}Rv$ resistant to various antituberculous agents

	Concentration of paromomycin (μg/ml)						
	0	20	50	100	200	500	1,000
Parent	+	+	+	+	−	−	−
SM-resistant*1	+	+	+	±	−	−	−
INH-resistant*2	+	+	+	−	−	−	−
PAS-resistant*3	+	+	−	−	−	−	−
KM-resistant*4	+	+	+	+	+	+	+
LVM-resistant*5	+	+	+	+	+	+	±
CPM-resistant*6	+	+	+	+	+	±	−
VM-resistant*7	+	+	+	−	−	−	−
TH-resistant*8	+	+	+	−	−	−	−
EB-resistant*9	+	+	+	−	−	−	−
RFP-resistant*10	+	+	+	−	−	−	−

*1 Resistant to 1,000 μg/ml or more streptomycin;
*2 Resistant to 100 μg/ml isoniazid;
*3 Resistant to 1,000 μg/ml sodium p-aminosalicylate;
*4 Resistant to 1,000 μg/ml or more kanamycin;
*5 Resistant to 800 μg/ml or more lividomycin;
*6 Resistant to 200 μg/ml capreomycin;
*7 Resistant to 100 μg/ml viomycin;
*8 Resistant to 100 μg/ml ethionamide;
*9 Resistant to 5 μg/ml ethambutol;
*10 Resistant to 1,000 μg/ml or more rifampicin.

The degree of resistance was measured by the "Actual count" method using a small inoculum consisting of 20 to 100 viable units.

Table 2. Susceptibility to paromomycin of *M. tuberculosis* Aoyama-B resistant to various antituberculous agents

	Concentration of paromomycin (μg/ml)						
	0	20	50	100	200	500	1,000
Parent	+	+	+	+	−	−	−
SM-resistant*1	+	+	+	+	−	−	−
INH-resistant*2	+	+	+	+	−	−	−
PAS-resistant*3	+	+	+	−	−	−	−
KM-resistant*4	+	+	+	+	+	+	+
LVM-resistant*5	+	+	+	+	+	+	+
CPM-resistant*6	+	+	+	+	+	+	+
VM-resistant*7	+	+	+	+	−	−	−
TH-resistant*8	+	+	+	−	−	−	−
EB-resistant*9	+	+	+	−	−	−	−
RFP-resistant*10	+	+	+	+	−	−	−

*1 Resistant to 1,000 μg/ml or more streptomycin;
*2 Resistant to 1,000 μg/ml isoniazid;
*3 Resistant to 1,000 μg/ml sodium p-aminosalicylate;
*4 Resistant to 1,000 μg/ml or more kanamycin;
*5 Resistant to 800 μg/ml or more lividomycin;
*6 Resistant to 200 μg/ml capreomycin;
*7 Resistant to 100 μg/ml viomycin;
*8 Resistant to 100 μg/ml ethionamide;
*9 Resistant to 10 μg/ml ethambutol;
*10 Resistant to 1,000 μg/ml or more rifampicin.

The degree of resistance was measured by the "Actual count" method using a small inoculum consisting of 20 to 100 viable units.

Fig. 2. Cross-resistance-relationships between paromomycin (PM), lividomycin (LVM), kanamycin (KM), capreomycin (CPM), and viomycin (VM) resistances in *M. tuberculosis* $H_{37}Rv$ and Aoyama-B.

The figure shows the cross-resistance-relationships in mutant strains isolated by multi-step selection (multi-step transfers on drug-containing media). Cross-resistance-relationships in respect to lividomycin, kanamycin, capreomycin, and viomycin have been cited from previous works[11,12].

Arrow (A to B) indicates that a mutant resistant to A is resistant to B. Dotted line indicates that cross-resistance occurs not always but occasionally.

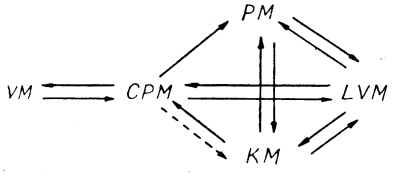


Table 3. Susceptibility of *M. tuberculosis* $H_{37}Rv$ and Aoyama-B resistant to paromomycin to various antituberculous agents

Antituberculous agent	Degree of resistance measured by the "actual count" method (μg/ml)			
	M. tuberculosis $H_{37}Rv$ parent	PM-resistant*	*M. tuberculosis* Aoyama-B parent	PM-resistant**
SM	4	8	4	8
INH	0.02	0.02	0.02	0.02
PAS	0.04	0.04	0.04	0.04
KM	16	>500	16	>500
LVM	63	>500	63	>500
CPM	16	16	16	16
VM	16	16	16	16
TH	8	8	16	16
EB	2	2	2	2
RFP	4	4	8	8
PM	100	>2,000	100	>2,000

* Resistant to 2,000 μg/ml or more paromomycin.
** Resistant to 2,000 μg/ml or more paromomycin.
The concentrations used are 500, 250, 125, 63, 32, 16, 8, 4, 2 and 0 μg/ml for SM, KM, LVM, CPM, VM, TH, EB and RFP, and are 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, 0.04, 0.02 and 0 μg/ml for INH and PAS.

paromomycin-resistant strains are shown in Tables 1 to 3.

Kanamycin-resistant, lividomycin-resistant, and capreomycin-resistant strains were resistant to paromomycin. On the other hand, paromomycin-resistant strains were resistant to kanamycin and lividomycin, but not resistant to capreomycin. The kanamycin-resistant strains used in these experiments are the strains obtained by multi-step selection. The summary of the results is shown in Figure 2.

Cross-resistance-relationships shown in the above were obtained in resistant strains isolated by multi-step selection (multi-transfers on drug-containing media). However, the results obtained in multi-step resistant strains might not be a direct reflex of gene action, although it may provide a possible feature in clinical use. They can contain some complex factors. It is considered that cross-resistance-relationship as a direct reflex of gene action will be obtained rather in resistant mutants obtainable by one-step-selection.

Thus, various resistant strains of *M. tuberculosis* $H_{37}Rv$ were isolated by one-step-selection and tested for their resistance to other agents (Table 4). As it is known that kanamycin-highly resistant strains and kanamycin-lowly resistant strains show different

Table 4. Cross-resistance-relationships of *M. tuberculosis* $H_{37}Rv$ between paromomycin (PM), lividomycin (LVM), kanamycin (KM) and capreomycin (CPM) resistances.

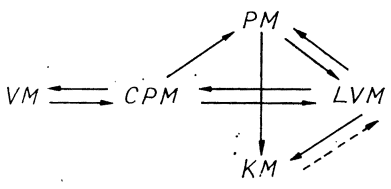
Strain	Concentration of antituberculous agent (μg/ml)												
		KM				LMV		CPM		PM			
	0	100	200	500	1,000	200	400	100	200	200	500	1,000	2,000
PM 1,000 Ra	+	+	+	+	+	+	+	−	−	+	+	+	+
PM 1,000 Rb	+	+	+	−	−	+	+	−	−	+	+	+	+
PM 1,000 Rc	+	+	+	−	−	+	+	−	−	+	+	+	+
PM 1,000 Rd	+	+	+	+	+	+	+	−	−	+	+	+	+
PM 1,000 Re	+	+	+	+	+	+	+	−	−	+	+	+	+
PM 2,000 Ra	+	+	+	+	+	+	+	−	−	+	+	+	+
PM 2,000 Rb	+	+	+	+	+	+	+	−	−	+	+	+	+
KM 200 Ra	+	+	+	−	−	+	+	−	−	−	−	−	−
KM 200 Rb	+	+	+	−	−	−	−	−	−	−	−	−	−
KM 200 Rc	+	+	+	−	−	+	+	+	−	−	−	−	−
KM 200 Rd	+	+	+	−	−	−	−	−	−	−	−	−	−
KM 200 Re	+	+	+	−	−	−	−	−	−	−	−	−	−
KM 500 Ra	+	+	+	+	+	+	+	−	−	+	−	−	−
KM 500 Rb	+	+	+	+	+	+	+	−	−	+	−	−	−
KM 500 Rc	+	+	+	+	+	+	+	−	−	+	+	−	−
KM 500 Rd	+	+	+	−	−	−	−	−	−	−	−	−	−
KM 500 Re	+	+	+	−	−	−	−	−	−	−	−	−	−
CPM 200 R	+	−	−	−	−	+	+	+	+	+	+	+	+
LVM 400 R	+	+	+	+	+	+	+	+	+	+	+	+	+

The degree of resistance was measured by the "actual count" method using a small inoculum consisting of 20 to 100 viable units. Growth was read at 4 weeks.

Fig. 3. Cross-resistance-relationships between lividomycin (LVM), paromomycin (PM), kanamycin (KM), capreomycin (CPM), and viomycin (VM) resistances in *M. tuberculosis* $H_{37}Rv$.

The figure shows the cross-resistance-relationships in resistant mutants obtainable by one-step-selection.

Arrow (A to B) indicates that a mutant resistant to A is resistant to B. Dotted arrow indicates that cross-resistance is seen not in lowly resistant mutants but in highly resistant mutants.



characters in relation to cross-resistance-relationship[11,12], kanamycin-resistant strains were isolated by two different concentrations, 200 μg/ml and 500 μg/ml.

The results showed that paromomycin-resistant strains were resistant to lividomycin and kanamycin, but susceptible to capreomycin (Table 4).

Strains showing a low resistance to kanamycin are susceptible to paromomycin and capreomycin, and usually susceptible to lividomycin, too. On the other hand, strains showing a high level of kanamycin resistance remained susceptible to paromomycin, though they were slightly more resistant to it (Table 4). The strains were susceptible to capreomycin, but resistant to lividomycin.

Capreomycin-resistant strains was resistant to lividomycin and paromomycin. Lividomycin-resistant strains were resistant to kanamycin, capreomycin and paromomycin (Table 4).

The results are summarized as shown in Figure 3.

Table 5. Cross-resistance-relationships in *M. tuberculosis* isolated from patients not treated previously with any drug (part 1)

Strain		Concentration of antituberculous agent (μg/ml)									
		Control	Lividomycin			Kanamycin			Capreomycin		
		0	200	400	800	100	200	500	50	100	200
L-2	Parent	+	−	−	−	−	−	−	−	−	−
	LVM 200 R	+	+	+	+	+	+	+	+	+	+
	KM 100 R	+	−	−	−	+	+	−	+	−	−
	KM 200 Ra	+	−	−	−	+	+	−	−	−	−
	KM 200 Rb	+	−	−	−	+	+	−	−	−	−
	CPM 100 R	+	−	−	−	−	−	−	+	+	+
	CPM 200 R	+	−	−	−	−	−	−	+	+	+
L-3	Parent	+	−	−	−	−	−	−	−	−	−
	LVM 200 R	+	+	+	+	+	+	+	+	+	+
	KM 100 R	+	−	−	−	+	+	−	−	−	−
	CPM 200 R	+	−	−	−	−	−	−	+	+	+
L-4	Parent	+	−	−	−	−	−	−	−	−	−
	KM 100 R	+	−	−	−	+	+	−	−	−	−
	KM 200 R	+	−	−	−	+	+	−	−	−	−
	CPM 200 Ra	+	−	−	−	−	−	−	+	+	+
	CPM 200 Rb	+	−	−	−	−	−	−	+	+	+
L-7	Parent	+	−	−	−	−	−	−	−	−	−
	KM 200 Ra	+	−	−	−	+	+	−	−	−	−
	KM 200 Rb	+	−	−	−	+	+	−	−	−	−
L-8	Parent	+	−	−	−	−	−	−	−	−	−
	KM 100 Ra	+	−	−	−	+	+	−	−	−	−
	KM 100 Rb	+	−	−	−	+	+	−	−	−	−
	CPM 50 Ra	+	−	−	−	−	−	−	+	+	+
	CPM 50 Rb	+	−	−	−	−	−	−	+	+	+
L-9	Parent	+	−	−	−	−	−	−	−	−	−
	KM 100 R	+	−	−	−	+	+	−	−	−	−
	CPM 50 Ra	+	−	−	−	−	−	−	+	+	+
	CPM 50 Rb	+	−	−	−	−	−	−	+	+	+
	CPM 100 R	+	−	−	−	−	−	−	+	+	+

The degree of resistance was measured by the "actual count" method using a small inoculum consisting of 20 to 100 viable units.

Cross-resistance-relationships between lividomycin, kamamycin, and capreomycin resistances of *M. tuberculosis* freshly isolated from patients

Cross-resistance-relationships in tubercle bacilli isolated freshly from patients are shown in Tables 5 and 6. The results obtained in "wild" strains of *M. tuberculosis*, which were isolated from patients previously not treated with any antituberculous agent, were slightly different from the results obtained in *M. tuberculosis* $H_{37}Rv$. Mutant strains resistant to a low concentration of kanamycin were susceptible to lividomycin and capreomycin. Strains resistant to capreomycin were susceptible to kanamycin and lividomycin. However, there were a few exceptions. As seen in strain L-13, some strains with a low degree of kanamycin resistance could be resistant to capreomycin, and, as seen in strain L-16, some strains could be resistant to livido-

Table 6. Cross-resistance-relationships in *M. tuberculosis* isolated from patients not treated previously with any drug (part 2)

	Concentration of antituberculous agent (μg/ml)									
	Control	Lividomycin			Kanamycin			Capreomycin		
	0	200	400	800	100	200	500	50	100	200
L-10 Parent	+	−	−	−	−	−	−	−	−	−
KM 100 R	+	+	+	+	+	+	−	−	−	−
KM 100-500Ra	+	+	+	+	+	+	+	−	−	−
KM 100-500Rb	+	+	+	+	+	+	+	−	−	−
L-13 Parent	+	−	−	−	−	−	−	−	−	−
KM 100 R	+	−	−	−	+	+	−	+	+	+
L-14 Parent	+	−	−	−	−	−	−	−	−	−
KM 100 Ra	+	−	−	−	+	+	−	−	−	−
KM 100 Rb	+	−	−	−	+	+	−	−	−	−
KM 100 Rc	+	−	−	−	+	+	−	−	−	−
CPM 50 Ra	+	−	−	−	−	−	−	+	+	+
CPM 50 Rb	+	−	−	−	−	−	−	+	+	+
CPM 50 Rc	+	−	−	−	−	−	−	+	+	+
CPM 100 Ra	+	−	−	−	−	−	−	+	+	+
CPM 100 Rb	+	−	−	−	−	−	−	+	+	+
CPM 100 Rc	+	−	−	−	−	−	−	+	+	+
L-15 Parent	+	−	−	−	−	−	−	−	−	−
LVM 200 R	+	+	+	+	+	+	+	+	+	+
L-16 Parent	+	−	−	−	−	−	−	−	−	−
KM 100 Ra	+	+	+	+	+	+	−	−	−	−
KM 100 Rb	+	+	+	+	+	+	−	−	−	−

KM 100-500 Ra, b are the strains obtained by two step-selection, the first at 100 μg/ml kanamycin and the second at 500 μg/ml kanamycin. The degree of resistance was measured by the "actual count" method using a small inoculum consisting of 20 to 100 viable units.

mycin. However, capreomycin-resistant strains remained always susceptible to kanamycin and lividomycin.

The results obtained are summarized as shown in Figure 4.

The fact that capreomycin-resistant strains remain susceptible to kanamycin agrees with our previous observation that tubercle bacilli resistant to a high concentration of kanamycin, which were isolated from patients treated with kanamycin, were resistant to capreomycin, but those resistant to a low concentration of kanamycin remained susceptible to capreomycin[13]. Capreomycin-resistant tubercle bacilli were susceptible to kanamycin[13].

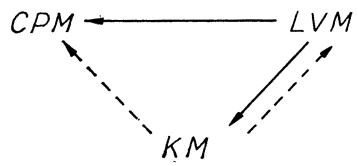
The fact that kanamycin-resistant strains obtained by multi-step selection are resistant to capreomycin and those obtained by one-step selection are not resistant to it seems to be explained as follows. Kanamycin-resistant mutants essentially are susceptible to capreomycin. However, as seen in strain L-13 (Table 6), kanamycin-resistant mutants that are resistant to capreomycin occasionally can appear probably as a result of Complex mutation[14]. Such mutants may have more profit for survival in the presence of kanamycin and hence the ratio of these mutants increases in a whole bacterial population.

Comparing the chemical structure of the antibiotics to each other, a rule seems to exist in development of resistance. A hypothesis that development of resistance to an aminoglucoside antibiotic is produced by adaptation of organism to a whole structure of an antibiotic seems to explain the cross-resistance-relationships observed. Development of resistance to lividomycin, an antibiotic that has the

Fig. 4. Cross-resistance-relationships between lividomycin (LVM), kanamycin (KM) and capreomycin (CPM) resistances in 'wild' strains of *M. tuberculosis* freshly isolated from patients.

The figure shows the cross-resistance-relationships in resistant mutants obtainable by one-step-selection.

Arrow (A to B) indicates that a mutant resistant to A is resistant to B. Dotted arrow indicates that cross-resistance occurs occasionally.



most complicated structure, causes resistance to antibiotics which have a simpler structure, for example, kanamycin. On the other hand, development of resistance to an antibiotic with simple structure, for example, kanamycin, does not cause resistance to antibiotics with a more complicated structure, for example, lividomycin and paromomycin.

The results obtained suggest that prior use of kanamycin in treatment of tuberculosis may cause *a priori* capreomycin resistance, but not *vice versa*. After the use of kanamycin, clinical effectiveness of capreomycin is no more expected. On the other hand, even after the use of capreomycin, clinical effectiveness of kanamycin is expected.

Summary

1. Cross-resistance relationships between paromomycin, lividomycin, kanamycin, and capreomycin resistances in *Mycobacterium tuberculosis* were studied. The results obtained in one-step resistant mutants are as follows.

(a) Strains resistant to paromomycin are resistant to kanamycin and lividomycin, but remain susceptible to capreomycin.

(b) Strains resistant to lividomycin are resistant to paromomycin, kanamycin, and capreomycin.

(c) Strains resistant to high concentrations of kanamycin (500 μg/ml or more in OGAWA egg medium) usually are resistant to lividomycin, but are susceptible to paromomycin and capreomycin. Strains resistant to low concentrations of kanamycin (200 μg/ml or less in OGAWA egg medium) usually are susceptible to all paromomycin, lividomycin, and capreomycin, although they can occasionally be resistant to capreomycin or lividomycin.

(d) Strains resistant to capreomycin are resistant to lividomycin and paromomycin, but are susceptible to kanamycin.

2. Strains resistant to high concentrations of kanamycin obtained by multi-step selection are resistant to lividomycin, paromomycin, and capreomycin.

3. Pattern of paromomycin resistance of *M. tuberculosis* ($H_{37}Rv$) is a single-step pattern, and resistant mutants show only one phenotype of resistance.

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