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Activity of Minocycline Against R Factor-Carrying *Enterobacteriaceae*[1]

H. JAROLMEN, D. HEWEL, AND E. KAIN
American Cyanamid Company, Agricultural Division, Princeton, New Jersey 08540

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Minocycline (Min) and other tetracyclines were tested in vivo and in vitro for activity against strains of salmonellae harboring R factor-mediated chlortetracycline (A) resistance. Minocycline was more active in vitro against these strains than the other tetracyclines. Mice infected with these cultures responded to treatment with Min, but there was little or no response to treatment with the other tetracyclines. Various A-resistant field isolates of *Salmonella typhimurium* and *Escherichia coli* were all shown to be more susceptible in vitro to Min than to A. Several of these cultures were shown to transfer A resistance. Cultures of normal bacterial flora were isolated from swine fecal matter after plating on Min-supplemented MacConkey agar. All recovered cultures were more susceptible in vitro to Min than to A. Several isolates were able simultaneously to transfer Min and A resistances into *S. choleraesuis* var. *kunzendorf*; however, the levels of Min resistance in either the donors or exconjugants were no higher than in those cultures of salmonellae which responded in vivo to Min therapy. The same was true for the levels of Min resistance in the field isolates of *S. typhimurium* and *E. coli*. The increased potency of Min, as compared to tetracycline, against R factor-carrying bacteria was not observed for organisms made resistant (presumably chromosomally) to A by successive transfers in increasing concentrations of the agent, suggesting different mechanisms as the basis for chromosomal versus R factor-mediated A resistances.

Minocycline (Min) is a semisynthetic tetracycline antibiotic. Redin (3) found it to be a unique tetracycline in that it was active both in vitro and in mice against strains of staphylococci that were resistant to other tetracyclines. The present work was undertaken to test Min and to compare it with other tetracyclines for in vitro and in vivo activity against R factor-carrying *Enterobacteriaceae*. Min was initially employed in this work to study chromosomal versus R factor-mediated chlortetracycline (A) resistances.

MATERIALS AND METHODS

Organisms. A description of the bacterial strains used is given in Table 1.

Media. Liquid cultures were prepared in Penassay (PA) Broth (Difco). All cultures were routinely tested for antibiotic susceptibility by the paper disc method on PA and Mueller Hinton Agar (Difco). All sensitivity discs were obtained from BBL. MacConkey agar was used in all media selective for recipients with acquired transmissible resistances. The levels of drug resistance were determined in PA Broth by a tube dilution technique.

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Drugs. The drugs used were chlortetracycline hydrochloride (A), demethylchlortetracycline (Dec), minocycline hydrochloride (Min), sulfaethoxypyridazine (SE), tetracycline hydrochloride (TET; American Cyanamid Co.); doxycycline hyclate, oxytetracycline hydrochloride, Rondomycin (Chas. Pfizer & Co., Inc.); dihydrostreptomycin sulfate (DS; E. R. Squibb & Sons); and nalidixic acid (NA; Sterling-Winthrop Research Institute).

Transfer of drug resistance in vitro. The technique used for determination of conjugal transfer of R factors in vitro was that of Watanabe (4). Concentrations of each drug incorporated in the media selective for R factor-carrying (R^+) recipients were (in μg/ml: NA, 100; A, 25; SE, 500; DS, 10; and Min, 10 and 25.

Drug sensitivity tests. The disc method was described in a previous paper (2). For the tube dilution method, dilutions of the various antibiotics were made in PA Broth which was then inoculated with 0.1 ml of a 10^{-5} dilution of an overnight (18 hr) culture of each strain tested [approximately 10^8 colony-forming units (CFU)]. The lowest concentration of the antibiotic which prevented visible growth was taken as the minimal inhibitory concentration (MIC).

Swiss Webster female mice (Taconic Farms) weighing 22 $\pm$ 2 g were used.

Infection and medication. Each mouse was injected intraperitoneally with 0.5 ml of a 5-hr test culture

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(various dilutions in saline) by using 25-gauge needles attached to 2.5-ml syringes. Drugs were administered in the feed beginning 3 hr before intraperitoneal infection and continued for 7 days postinfection; mice were maintained on antibiotic-free commercial mouse feed thereafter through 21 days postinfection. Mortality was recorded twice daily during the 21-day trial period. Data summarized in Tables 6 and 7 reflect the status of animals 14 days after infection, and these numbers were used as the basis for the calculations. Drug effects for all in vivo experiments reported can be seen more clearly at 14 days than at 21 days.

TABLE 1. *Description of bacterial strains used*

Strain	Description[a]
Salmonella choleraesuis var. *kunzendorf*	
RC221NA	Competent NA-resistant recipient of R factors; virulent for mice and pigs (1, 2)
437 L.I.	Smooth virulent RC221NA with an R factor (A DS) recovered from the large intestine of pig 437, which was infected with RC221NA
RC221NA-100	Resistant mutant selected after serial transfers of RC221NA in increasing levels of A
460 S.I.-2	Rough variant without an R factor isolated from the small intestine of pig 460, which was infected with RC221NA (1); highly competent recipient for R factors with reduced virulence for mice.
460 EK-1	Rough variant with an R factor (A DS) recovered from the small intestine of pig 460 infected with RC221NA; reduced in virulence
405 L.I.	Smooth virulent RC221NA with an R factor (A DS) recovered from the large intestine of pig 405 infected with RC221NA
Salmonella typhimurium	
4399–68 and 4354-68	From the Communicable Disease Center, Atlanta, Ga.
H-S	Isolate from tissue of a calf succumbing to salmonellosis
Salmonella dublin	
298-68	From the Communicable Disease Center, Atlanta, Ga.
Escherichia coli	
K-12NA	NA-resistant culture of *E. coli* K-12 (1, 2)
ADJ-100	K-12NA which first received an R factor (AM A) from an *E. coli* of pig origin and then subsequently received additional transferable resistances (DS Su) from a rough RC221NA pig isolate; it forms granular growth on the bottom of the tube in liquid medium (leaving the broth transparent)
DMJ-100	Resistant mutant selected after serial transfers of K-12NA in increasing levels of A
L-1260	Submitted by T. L. Landers
MD-14; MD-21; MD-22	Submitted by T. L. Landers; cultures were isolated from separate outbreaks of severe air sac disease which caused high mortality in affected broiler flocks; isolates were made from the pericardial sac immediately after the bird was killed

[a] Abbreviations: A, chlortetracycline; AM, ampicillin; DS, dihydrostreptomycin; NA, nalidixic acid; Su, sulfonamides.

RESULTS AND DISCUSSION

In vitro susceptibility to Min and A of bacterial field isolates. Seven A-resistant field isolates of *Salmonella typhimurium* and *Escherichia coli* were all shown to be more susceptible in vitro to Min than to A (Table 2). Four of these isolates were shown to transfer A resistance by using either K-12NA or 460 S.I.-2 as recipient. *S. dublin* strain 298–68, which does not harbor a transferable R factor (R^-), was equally sensitive to both agents.

Levels of Min and A resistance of strain 460 S.I.-2 with R factors of pig origin. An experiment was designed to measure the levels of Min and A resistance of recipient strain 460 S.I.-2 before and after receiving R factors which conferred A resistance. In this study, enteric organisms isolated from healthy pigs were used as donors of R factors. For this purpose, duplicate fecal swabs ob-

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TABLE 2. *In vitro susceptibility to minocycline (Min) and chlortetracycline (A) of bacterial field isolates with and without R factor-mediated A resistance*

Culture	Strain designation	Antibiotic resistance pattern[a]	A resistance transferability	Minimal inhibitory concn (μg/ml)	
				Min	A
Salmonella typhimurium	4399–68	AM A CM DS K N Su	+	31.2	125.0
	4354–68	AM A DS Su	+	3.9	31.2
	H-S	AM A DS	Not done	2.0	62.5
S. dublin	298–68	Sensitive	−	2.0	2.0
E. coli	L-1260	A DS K N Su	−	7.8	62.5
	MD-14	A DS	−	7.8	62.5
	MD-21	A DS	+	3.9	62.5
	MD-22	A DS	+	7.8	125.0

[a] AM, ampicillin; CM, chloramphenicol; DS, dihydrostreptomycin; K, kanamycin; N, neomycin; Su, sulfonamides.

TABLE 3. *Minocycline (Min) and chlortetracycline (A) resistances of Salmonella choleraesuis var. kunzendorf strain 460 S.I.-2 with and without R factors from various Enterobacteriaceae recovered from pig feces*

Donor	Minimal inhibitory concn (μg/ml)				Media used to select for exconjugant
	Donor		Recipient 460 S.I.-2 (after transfer from respective donor)[a]		
	Min	A	Min	A	
53–3–3	31.2	125.0	31.2	250.0	A + NA[b]
			31.2	250.0	Min + NA
53–3–7	15.6	125.0	31.2	250.0	A + NA
			31.2	250.0	Min + NA
53–3–10	7.8	125.0	31.2	125.0	A + NA
			31.2	125.0	Min + NA
53–3–12	15.6	125.0	31.2	250.0	A + NA
			31.2	250.0	Min + NA
53–9–2	15.6	125.0	31.2	250.0	A + NA
			31.2	250.0	Min + NA

[a] Before transfer, the minimal inhibitory concentrations of Min and A were 2 for recipient 460 S.I.-2.
[b] NA, nalidixic acid.

tained from five pigs were plated directly to MacConkey agar supplemented with Min at 50 μg/ml. Relatively few colonies, which were restricted in size, grew on this medium, whereas confluent growth appeared on nonsupplemented medium from all swabs. Five of 18 isolates of *Enterobacteriaceae*, which were recovered from Min-supplemented medium, were shown to transfer Min and A resistances simultaneously into recipient strain 460 S.I.-2. The five donor pig isolates and strain 460 S.I.-2, which had received R factors from these donors, were 4- to 16-fold more susceptible in vitro to Min than to A (Table 3). R factor-carrying recipients recovered from A selective medium had the same levels of resistance to Min and A as R factor-carrying recipients recovered from Min selective medium. The number of converted recipients appearing on each of these two selective media used (A plus NA and Min plus NA) were equivalent. It appears probable that a single resistance determinant governs both A and Min resistances.

In vitro susceptibility to Min and Tet of cultures with chromosomal or R factor-mediated A resistance. Cultures which were chromosomally or episomally resistant to A were derived from A-sensitive cultures of *E. coli* K-12 and *S. choleraesuis* var. *kunzendorf* RC221NA. These cultures were compared for their relative susceptibilities in vitro to Min and Tet. Min was more effective than Tet against R factor-carrying strains ADJ-100, 437 L.I., and 460 EK-1 (Table 4). This in-

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TABLE 4. *In vitro susceptibility to minocycline (Min) and tetracycline (Tet) of cultures with chromosomal or R factor-mediated chlortetracycline (A) resistance*

Culture	A Resistance		Minimal inhibitory concn (μg/ml)	
	R factor-mediated	Selected by serial transfer in A-containing media	Min	Tet
Escherichia coli K-12NA				
Parental	−	−	1.0	<0.5
ADJ-100	+	−	15.6	62.5
DMJ-100	−	+	15.6	7.8
Salmonella choleraesuis var. *kunzendorf* RC221NA				
Parental	−	−	2.0	1.0
437 L.I.	+	−	31.2	250.0
RC221NA-100	−	+	31.2	7.8
460 S.I.-2	−	−	2.0	1.0
460 EK-1	+	−	31.2	250.0

creased potency of Min as compared to Tet was not observed for cultures DMJ-100 and RC221-NA-100, which were made resistant (presumably chromosomally) to A by serial transfers of the sensitive parental cultures in increasing concentrations of the agent. This observation suggests there may be different mechanisms governing chromosomal versus R factor-mediated A resistances.

In vitro susceptibilities to various tetracyclines of enteric organisms harboring R factor-mediated A resistance. Three R^+ cultures were tested for in vitro susceptibility to seven tetracyclines (Table 5). *S. choleraesuis* var. *kunzendorf* strain 437 L.I., *S. typhimurium* strain 4399-68, and *E. coli* strain MD-22 were all shown to be more susceptible to Min than to six other tetracyclines.

In vivo susceptibility to Min and A of S. choleraesuis var. kunzendorf strain RC221NA and virulent R^+ pig isolates of RC221NA. In a single trial, A and Min were evaluated against experimental *S. choleraesuis* infections in mice (Table 6). Parental strain RC221NA (R^-) and two virulent isolates of RC221NA, 437 L.I. and 405 L.I., which have been shown to contain transmissible resistance determinants for A and DS, were used for the infections. Mice infected with parental strain RC221NA responded to therapy with both Min and A. Mice infected with 437 L.I. or 405 L.I. responded to treatment with Min but there was little response to A treatment.

TABLE 5. *In vitro susceptibilities to various tetracyclines of enteric organisms harboring R factor-mediated chlortetracycline resistance*

Drug	Minimal inhibitory concn (μg/ml)		
	S. choleraesuis var. *kunzendorf* strain 437 L.I.	*S. typhimurium* strain 4399-68	*E. coli* strain MD-22
Minocycline	15.6	15.6	7.8
Chlortetracycline	125.0	125.0	62.5
Tetracycline	250.0	125.0	125.0
Oxytetracycline	250.0	250.0	250.0
Rondomycin	125.0	125.0	62.5
Demethylchlortetracycline	62.5	62.5	31.2
Doxycycline	62.5	62.5	31.2

In vivo susceptibility to Min and other tetracyclines of S. typhimurium strain 4399-68 (R^+). A preliminary test was conducted in which mice were infected with *S. typhimurium* strain 4399-68 (R^+). At the infective dose used (3.9×10^5 CFU per mouse), none of the treatments was effective. In this trial, the mean survival time (MST) of the infected-untreated controls was 3.7 days. The highest levels of the drugs used in the diet (A at 4,000, Min at 1,000, and Dec at 4,000 μg/g) were ineffective either in preventing mortality or extending the MST.

In subsequent tests (Table 7), Min was much more effective than A or Dec in preventing mortality and extending the MST, when lower infective doses of strain 4399-68 were used. The median effective dose (95% confidence limits) calculated for Min from the dose-effect data of Table 7 was 161 (104-249) mg per kg per day, which was achieved with 1,000 μg/g in the diet. Neither A nor Dec was effective at the highest test level (4,000 μg/g).

Thus far, all of the cultures of *Enterobacteriaceae* which we have examined were sensitive to therapeutically achievable concentrations of minocycline.

In summary, Min has been shown to be a unique tetracycline in that it is active both in vitro and in mice against a variety of tetracycline-resistant R factor-carrying *Enterobacteriaceae*.

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TABLE 6. *In vivo susceptibility to minocycline (Min) and chlortetracycline (A) of Salmonella choleraesuis var. kunzendorf strain RC221NA with and without R factors*

Culture[a]	Drug	Drug concn in diet (μg/g)	Avg drug consumed (mg per kg per day)[b]	Survivors/total (14 days postinfection)	MST[c]
RC221NA	Min	2,000	348.7	9/10	
		1,000	174.8	10/10	
		500	81.4	10/10	
		250	42.4	10/10	
	A	4,000	651.1	7/10	13.3
		2,000	311.0	8/10	
		1,000	162.0	7/10	13.3
		500	101.4	8/10	
	Untreated			0/10	6.1
437 L.I.	Min	2,000	348.7	10/10	
		1,000	157.8	9/10	
		500	100.0	7/10	12.0
		250	39.5	8/10	
	A	4,000	683.8	4/10	6.0
		2,000	378.4	1/10	7.1
		1,000	191.2	2/10	7.4
		500	84.1	1/10	7.4
	Untreated			3/10	6.7
405 L.I.	Min	2,000	338.4	10/10	
		1,000	169.0	10/10	
		500	87.0	7/10	13.7
		250	45.4	7/10	13.0
	A	4,000	614.6	5/10	7.6
		2,000	339.4	2/9	7.7
		1,000	183.5	2/10	8.1
		500	88.3	2/10	6.6
	Untreated			0/10	8.3
Uninfected	Untreated			30/30	

[a] Infective doses of cultures RC221NA, 437 L.I., and 405 L.I. were 1.8×10^5, 5.0×10^4, and 4.8×10^4 CFU/mouse, respectively.

[b] Average daily drug consumption in milligrams per kilogram of mouse weight for the first 2 days of medication.

[c] Mean survival time (days) of mice that died through 14 days postinfection.

TABLE 7. *In vivo susceptibility to minocycline and other tetracyclines of R factor-carrying Salmonella typhimurium strain 4399-68*

Drug[a]	Drug concn in diet (μg/g)	Avg drug consumed (mg per kg per day)[b]	Survivors/total (14 days postinfection)[c]	Per cent survivors	Avg MST[d]
Min	2,000	286.1	19/30	63	12.1
	1,000	160.9	20/40	50	10.4
	500	77.0	13/40	32	8.7
	250	37.7	10/40	25	7.2
A	4,000	785.2	1/20	5	6.5
	2,000	352.9	1/20	5	6.1
	1,000	171.8	1/20	5	6.1
Dec	4,000	774.1	1/20	5	6.3
	2,000	359.8	2/20	10	5.6
	1,000	169.4	1/20	5	5.6
	500	85.0	1/20	5	6.1
Untreated			3/40	8	6.1
Uninfected-untreated			20/20	100	

[a] Min, minocycline; A, chlortetracycline; Dec, demethylchlortetracycline.

[b] Average daily drug consumption in milligrams per kilogram of body weight for the first 2 days of medication.

[c] Data were pooled from four tests. Infective doses of culture 4399-68 were 4.5×10^3, 4.5×10^2, 4.3×10^3, and 4.3×10^2 CFU/mouse for tests 1 through 4, respectively.

[d] Mean survival time (days) of mice that died through 14 days postinfection.

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