

Thiopurine Drugs Azathioprine and 6-Mercaptopurine Inhibit *Mycobacterium paratuberculosis* Growth In Vitro[∇]

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The *in vitro* susceptibility of human- and bovine-origin *Mycobacterium paratuberculosis* to the thiopurine drugs 6-mercaptopurine (6-MP) and azathioprine (AZA) was established using conventional plate counting methods and the MGIT 960 ParaTB culture system. Both 6-MP and AZA had antibacterial activity against *M. paratuberculosis*; isolates from Crohn's disease patients tended to be more susceptible than were bovine-origin isolates. Isolates of *Mycobacterium avium*, used as controls, were generally resistant to both AZA and 6-MP, even at high concentrations (≥ 64.0 $\mu\text{g/ml}$). Among rapidly growing mycobacteria, *Mycobacterium phlei* was susceptible to 6-MP and AZA whereas *Mycobacterium smegmatis* strains were not. AZA and 6-MP limited the growth of, but did not kill, *M. paratuberculosis* in a dose-dependent manner. Anti-inflammatory drugs in the sulfonamide family (sulfapyridine, sulfasalazine, and 5-aminosalicylic acid [mesalamine]) had little or no antibacterial activity against *M. paratuberculosis*. The conventional antibiotics azithromycin and ciprofloxacin, used as control drugs, were bactericidal for *M. paratuberculosis*, exerting their killing effects on the organism relatively quickly. Simultaneous exposure of *M. paratuberculosis* to 6-MP and ciprofloxacin resulted in significantly higher CFU than use of ciprofloxacin alone. These data may partially explain the paradoxical response of Crohn's disease patients infected with *M. paratuberculosis* to treatment with immunosuppressive thiopurine drugs, i.e., they do not worsen with anti-inflammatory treatment as would be expected with a microbiological etiologic pathogen. These findings also should influence the design of therapeutic trials to evaluate antibiotic treatments of Crohn's disease: AZA drugs may confound interpretation of data on therapeutic responses for both antibiotic-treated and control groups.

The etiology of Crohn's disease remains elusive, but current consensus opinion is that Crohn's disease results from the interplay of host genetics and one or more environmental triggers (7). Genetic markers for susceptibility to Crohn's disease have been discovered, notably the CARD15 (15, 21, 28) gene and most recently the interleukin-23r (10) and ATG16L1 (16, 32) genes. The increased incidence seen in many industrialized countries supports the existence of an environmental trigger for Crohn's disease (11, 23, 37, 39, 43). Bacterial pathogens fit a pathobiology model involving abnormal host response to infection resulting from genetic defects in gastrointestinal antigen processing.

One trigger for Crohn's disease may be *Mycobacterium paratuberculosis*, also referred to as *Mycobacterium avium* subsp. *paratuberculosis*, with or without host genetic influences that increase infection susceptibility or alter response to infection (9, 40). *M. paratuberculosis* causes a type of chronic inflammatory bowel disease in a wide array of ruminant species, as well as in nonhuman primates (5, 18). The infection is prevalent in domestic agriculture ruminants, and it has been diagnosed in wildlife species as well. Human and animal *M. paratuberculosis* isolates share common genotypes (12, 29). Exposure of humans to *M. paratuberculosis* could occur by both direct and indirect contamination of food and water (3, 4, 44).

The thiopurine drugs azathioprine (AZA; Imuran) and its metabolite 6-mercaptopurine (6-MP) are among the most commonly used immunosuppressive drugs used to treat Crohn's disease symptoms (42). They both induce and maintain Crohn's disease remission. Compounds related to 5-aminosalicylates (sulfasalazine [SS] and mesalamine) are other drugs used to suppress inflammation in Crohn's disease patients (17). One observation arguing against an involvement of *M. paratuberculosis*, or any other infectious agent, as a primary cause of Crohn's disease is that patients treated with immunosuppressive drugs do not clinically worsen (35).

Greenstein et al. demonstrated, however, that thiopurine drugs inhibit the growth of *M. paratuberculosis* (13). The goal of this study was to characterize 6-MP's effect on *M. paratuberculosis* growth in culture and contrast it with the antibacterial effects of conventional antimycobacterial antibiotics.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. A total of 11 bovine- and human-origin *M. paratuberculosis* strains were used in this study (Table 1). All strains were initially cultured in 7H9 broth supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson, Sparks, MD) and 2 $\mu\text{g/ml}$ of mycobactin J (Allied Monitor, Fayette, MO) for 1 month at 37°C. Seven *Mycobacterium avium* strains were used for comparison. These were cultured in 7H9 broth supplemented with 10% OADC (Becton Dickinson, Sparks, MD) for 2 weeks to 1 month at 37°C (Table 1).

Three representatives of rapidly growing mycobacteria were tested: *Mycobacterium phlei* ATCC 11758, *Mycobacterium smegmatis* ATCC 14468, and *M. smegmatis* mc²155. Two nonmycobacterial strains, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212, were included as commonly used drug susceptibility control organisms (Table 1).

The identity of all mycobacteria was verified by multiplex PCR for insertion

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TABLE 1. Bacterial strains tested in this study

Bacterial strain	Isolate source
<i>M. paratuberculosis</i>	
ATCC 19698	Bovine, clinical case of paratuberculosis, type strain
JTC303	Bovine, clinical case of paratuberculosis, JTC ^a
UCF-3	Human, Crohn's disease patient ileum, UCF ^b
UCF-4	Human, Crohn's disease patient ileum, UCF ^b
UCF-5	Human, Crohn's disease patient ileum, UCF ^b
UCF-7	Human, Crohn's disease patient ileum, UCF ^b
UCF-8	Human, Crohn's disease patient ileum, UCF ^b
B213	Bovine, clinical case of paratuberculosis, UCF ^b
B236	Bovine, clinical case of paratuberculosis, UCF ^b
B238	Bovine, clinical case of paratuberculosis, UCF ^b
B244	Bovine, clinical case of paratuberculosis, UCF ^b
<i>M. avium</i>	
ATCC 35712	Chicken, TMC701, ^c serotype 2
ATCC 25291	Chicken, liver, TMC724, serotype 2, type strain
104	Human, AIDS patient, serotype 1 ^d
JTC48627	Bison, fecal sample
JTC981	Bongo, fecal sample
EPA3	Water, WSLH ^e
WSLH1544	Water, WSLH
Rapidly growing mycobacteria	
<i>M. phlei</i> ATCC11758	TMC1458, type strain
<i>M. smegmatis</i> ATCC14468	TMC1546, suggested neotype
<i>M. smegmatis</i> mc ² 155 ^f	Transformably competent isolate of mc ² 6
Other bacterial species ^g	
<i>E. coli</i> ATCC 25922	Clinical isolate, FDA strain
<i>E. faecalis</i> ATCC 29212	Human urine

^a JTC, Johne's Testing Center, Madison, WI.
^b Saleh Naser, University of Central Florida (UCF), Orlando, FL.
^c Trudeau Mycobacterial Culture Collection.
^d Obtained from A. M. Talaat (University of Wisconsin-Madison; originally from Raul Barletta at the University of Nebraska).
^e WSLH, Wisconsin State Hygiene Laboratory.
^f Obtained from the American Type Culture Collection.
^g Quality control strains used in antibiotic susceptibility testing.

elements IS900, IS901, IS1311, and IS1245 (Johne's Testing Center, Madison, WI) as well as by high-pressure liquid chromatography (HPLC) of cell wall mycolic acids by a reference laboratory (Wisconsin State Laboratory of Hygiene, Madison, WI).

Single-cell suspensions of each strain were prepared as previously described with slight modifications (41). Tenfold serial dilutions from seed lots of each strain were plated on 7H10 agar, and single colonies were inoculated into MGIT ParaTB tubes (Becton Dickinson, Sparks, MD) to quantify the number of organisms per ml (38). Seed lots of each strain were then kept in small aliquots at -80°C until use. After plate counting, 10⁴ to 10⁶ CFU of each strain were inoculated to tubes of MGIT ParaTB medium to determine the average time to detection (TTD) in the absence of drugs. For *M. paratuberculosis* in MGIT ParaTB medium, the TTD is directly related to the number of organisms inoculated into each tube (38).

Drugs tested. All test drugs were obtained from Sigma-Aldrich Co., St. Louis, MO, in a chemically pure form (purity > 99.0%). The drugs were SS, 5-aminosalicylic acid (5-ASA), sulfapyridine (SP), rifampin (RIF), nalidixic acid (NAL), 6-MP, AZA, azithromycin (AZM), and ciprofloxacin (CIP). Lyophilized drugs were dissolved in appropriate diluents according to the manufacturer's instructions. The drugs SS, 5-ASA, SP, 6-MP, and AZA were dissolved in 0.05 M NaOH in 10 mM phosphate-buffered saline (PBS), RIF and NAL were dissolved in 10 mM PBS (pH 7.2), AZM was dissolved in 50% ethanol, and CIP was dissolved in 0.5 N HCl in 10 mM PBS. All stock drug solutions were freshly prepared for each experiment and filter sterilized using a 0.22-µm polycarbonate syringe filter (Millipore Corp., Bedford, MA).

MGIT 960 drug susceptibility testing. Methods published for *M. tuberculosis* and *M. avium* susceptibility testing (1, 2, 20, 22, 31, 34) were adapted as closely as possible; however, the significantly longer generation time of *M. paratuberculosis* compared to *M. tuberculosis* made this challenging. For this reason, we performed drug susceptibility studies in three phases. First, we evaluated our MGIT 960 antibacterial susceptibility testing method for *M. paratuberculosis* ATCC 19698 by comparing MGIT 960 results with those of conventional agar plate methods using drugs well characterized for antimycobacterial activity (phase I). Second, we evaluated the effects of thiopurine drugs and 5-aminosalicylates on *M. paratuberculosis* and *M. avium* growth in MGIT ParaTB medium in comparison to conventional bactericidal antibiotics (phase II). Next, we tested the reproducibility of the findings and expanded the study to include more strains and mycobacterial species (phase III). Lastly, we tested the effect of a combination of CIP and 6-MP over a range of achievable concentrations in tissue on the viability of *M. paratuberculosis* (phase IV). The methods for each of the four phases of study were as follows.

(i) **Phase I.** A single type strain of *M. paratuberculosis*, ATCC 19698, was tested at three MGIT ParaTB medium inoculum levels (10⁴, 10⁵, and 10⁶ CFU per MGIT ParaTB tube). The final concentrations of each drug tested ranged from 1.0 to 64.0 µg/ml.

(ii) **Phase II.** Phase II, designed based on the results of phase I, used seven clinical strains of *M. paratuberculosis* of bovine or human origin and one *M. avium* strain, ATCC 35712, as a control. Some drugs to which *M. paratuberculosis* was not susceptible in phase I, specifically the SS drug family (SS, 5-ASA, and SP), were excluded, and AZA, a prodrug of 6-MP, was added to the trial (19, 27).

(iii) **Phase III.** The reproducibility of findings in phase II was determined. Additional strains of *M. paratuberculosis* also were tested, along with three other species of mycobacteria. The thiopurine drug concentrations tested were the same as in phase II, but one higher concentration (128.0 µg/ml) was added for some strains (see Table 4).

(iv) **Phase IV.** The effect of combined CIP and 6-MP on *M. paratuberculosis* strain UCF-7 viability was determined over a range of concentrations (0, 2, 4, and 6 µg/ml) and drug exposure times (0, 3, 6, 9, and 12 days). The results for this drug combination for *M. paratuberculosis* counts were compared to those for each drug individually, the drug-free control, and the 1% (1:100 original inoculum dilution) drug-free control.

MGIT 960 methodology details. Serial dilutions of single-cell suspensions of each mycobacterial strain were prepared, and 100 µl was inoculated into MGIT ParaTB medium (Becton Dickinson, Sparks, MD). Each tube contained 7 ml of medium and a fluorescent indicator embedded in silicone on the bottom of the tube. To each tube was added 800 µl of MGIT ParaTB supplement (Becton Dickinson, Sparks, MD), 500 µl of egg yolk suspension (Becton Dickinson, Sparks, MD), and 100 µl of test drug, resulting in final concentrations of 0.5 to 64.0 µg/ml. Tubes were incubated at 37°C in a MGIT 960 instrument and removed when the instrument signaled them as being positive. Samples from all signal-positive tubes were subcultured on Trypticase soy agar plates with 5% sheep blood (Becton Dickinson, Sparks, MD) to check for contamination. Acid-fast staining (Ziehl-Neelsen) was also performed on smears made from each signal-positive tube to confirm the presence of mycobacteria. For *E. coli* and *E. faecalis*, the standard broth microdilution method was used for susceptibility tests (8).

Interpretation of susceptibility results. There are no interpretive criteria of MGIT 960 algorithms for antibacterial susceptibility test interpretation for mycobacteria other than *M. tuberculosis* and *M. avium*. We used a similar interpretation system with slight modifications (1, 2, 22, 33, 45). Briefly, positive control MGIT ParaTB tubes were inoculated with only the test organism and the relevant drug solvent (solvent control). For comparison, these drug-free solvent control vials were inoculated with a 1:100 dilution of the normal organism inoculum (designed to represent growth of 1% of the original bacterial population). All MGIT ParaTB medium tubes were inoculated in duplicate with specified numbers of test organisms. In phase I trials, three *M. paratuberculosis*

inoculum levels were tested (10^4 , 10^5 , and 10^6 CFU), and in phases II, III, and IV only 10^5 to 10^6 CFU were inoculated to each MGIT ParaTB tube.

The bacterial growth rate in each MGIT ParaTB tube was defined by TTD, i.e., the number of days the MGIT ParaTB tubes were incubated until they were determined to be signal positive by the MGIT 960 instrument (38). The baseline for *M. paratuberculosis* inhibition was defined as the day that the 1:100 dilution inoculum control became signal positive. Thus the lowest concentration for each test drug that inhibited growth was the concentration that produced a TTD greater than that of the 1:100 dilution control tube. The minimum concentrations of each drug that completely suppressed growth of the test organism in the MGIT 960 system, i.e., the tubes were never signal positive by the end of the experiment at 56 days postinoculation, were also reported. For MGIT ParaTB tubes inoculated with 10^6 CFU *M. paratuberculosis*, this usually occurred between days 5 and 7 of incubation.

If the positive control tube became signal positive earlier than incubation day 4, the *M. paratuberculosis* inoculum was considered too high. Similarly, if the 1:100 dilution inoculum control tube did not become signal positive within 8 days after the positive control, the tube was considered underinoculated. If either control tube criterion was not met, the test was considered invalid and the test was repeated. An uninoculated MGIT ParaTB tube was used as the negative control for every trial.

Agar plate counting method. Agar plate counting methods for drug susceptibility testing were performed with 7H10 medium supplemented with 10% OADC (Becton Dickinson, Sparks, MD) and 2 $\mu\text{g/ml}$ of mycobactin J (Allied Monitor, Fayette, MO) for phase I, phase III, and phase IV trials (26). At selected times after exposure to drugs, surviving *M. paratuberculosis* cells were quantified by conventional plate counting for all controls and every drug concentration for the 10^6 -CFU *M. paratuberculosis* inoculum in phase I. In phase III, plate counts were done for only two concentrations of each drug, 2.0 $\mu\text{g/ml}$ (low concentration) and 16.0 $\mu\text{g/ml}$ (high concentration).

Bacteria were mixed with each specific drug and drug concentration in MGIT ParaTB medium and incubated at 37°C. At 3 and 5 days postinoculation an aliquot (0.1 ml) was removed for plate count determinations (note that MGIT 960 instrument readings were not taken on these tubes; the tubes merely provided the medium in which the bacterium-drug interaction took place). Bacterial cells were harvested from MGIT tubes by centrifugation, resuspended in PBS, and homogenized to break up bacterial cell clumps by vortexing with glass beads (30). Then, 10-fold serial dilutions (10^0 to 10^{-6}) were made in PBS, and 100 μl of each dilution was plated in quadruplicate on 7H10 agar supplemented 10% OADC (Becton Dickinson, Sparks, MD) and 2 $\mu\text{g/ml}$ of mycobactin J (Allied Monitor, Fayette, MO). The numbers of CFU were determined by visual inspection after incubation of plates at 37°C for 8 weeks. The lowest concentration of each drug tested by the agar counting method that inhibited growth was defined as the lowest concentration of the drug that produced a 99% ($2 \log_{10}$) reduction in CFU. These concentrations were compared to those determined by MGIT 960 drug susceptibility testing.

Quality control. *M. paratuberculosis* strain ATCC 19698 was included in all experiments and used to test the ability of each new lot of MGIT ParaTB medium, growth supplement, egg yolk, and mycobactin J to support *M. paratuberculosis* growth. Also, *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used as controls to assure drug efficacy, as these agents have predictable antibiotic susceptibility patterns. Solvent controls were used with each drug to distinguish anti-*M. paratuberculosis* activity due to solvents themselves from the activity of the drugs, and uninoculated MGIT ParaTB medium controls served to detect any contamination by extraneous organisms.

RESULTS

Phase I. Inoculum size is a critical factor in drug susceptibility testing of slowly growing mycobacteria (31). Three *M. paratuberculosis* ATCC 19698 inocula initially tested were 10^4 , 10^5 , and 10^6 CFU/tube. The average TTDs for inoculum controls (drug solvent only) were 4.97, 7.89, and 10.76 days for 10^6 , 10^5 , and 10^4 CFU/tube, respectively. The 1:100 dilution inoculum controls became signal positive at 10.76, 14.32, 21.74 days on average, respectively. The drug solvents had no effect on TTD if $>10^2$ CFU *M. paratuberculosis* were inoculated (multiple comparison test, $P = 0.83$; data not shown). However, the 10^4 -CFU inoculum was excluded from subsequent analyses because of the long incubation time required for the 1:100

TABLE 2. Tested drugs in phase I experiment and their effect on *M. paratuberculosis* ATCC 19698, as determined by MGIT 960^a and agar plate counting methods

Drug	Drug family/type	Lowest concn ($\mu\text{g/ml}$) producing inhibition in comparison with 1:100 dilution control by:		Minimum drug concn resulting in no detectable growth of <i>M. paratuberculosis</i> ^b
		MGIT 960	Agar counting method	
AZM	Macrolide	4.0	4.0	16.0
CIP	Quinolone	1.0	1.0	32.0
NAL	Quinolone	>64.0	>64.0	>64.0
RIF	First-line tuberculosis drug	2.0	2.0	>64.0
SP	Sulfonamide	>64.0	>64.0	>64.0
SS	Sulfonamide	>64.0	>64.0	>64.0
5-ASA	Anti-inflammatory	>64.0	>64.0	>64.0
6-MP	Anti-inflammatory	1.0	1.0	32.0

^a Each tube was inoculated with 10^6 CFU bacteria.

^b Signal negative by the BACTEC MGIT 960 instrument up to 56 days postinoculation.

dilution control to become signal positive, especially in the face of drug solvents, notably 50% ethanol.

When the *M. paratuberculosis* inoculum was $\geq 10^5$ CFU/tube, there was good agreement between MGIT 960- and agar plate-determined drug effects, with previously reported mycobacterium MIC ranges for all drugs tested (Table 2). An *M. paratuberculosis* inoculum amount of 10^5 versus 10^6 CFU did not affect the drug effects for any compound with the exception of RIF (data not shown). While the concentration of RIF needed to inhibit *M. paratuberculosis* was low, the organism was able to grow to some extent even in the face of the highest RIF concentration tested, 64.0 $\mu\text{g/ml}$.

Growth inhibition was seen at the lowest concentration with CIP (1.0 $\mu\text{g/ml}$). For AZM the lowest growth-inhibitory concentration was 4.0 $\mu\text{g/ml}$, and *M. paratuberculosis* was highly resistant to NAL (inhibitory concentration $> 64.0 \mu\text{g/ml}$) (Table 2).

Among the immunomodulatory drugs, the SS drug family (SS, 5-ASA, and SP) showed no significant anti-*M. paratuberculosis* activity even at high concentrations (64.0 $\mu\text{g/ml}$). Unexpectedly, 6-MP showed anti-*M. paratuberculosis* activity similar in potency to that of CIP (lowest inhibitory concentration = 1.0 $\mu\text{g/ml}$) by the MGIT 960 drug susceptibility test (Table 2).

Phase II. The susceptibility of *M. paratuberculosis* to 6-MP was confirmed, and similar results were found for its precursor drug, AZA (Table 3). Across the seven *M. paratuberculosis* strains tested, growth inhibition for 6-MP was comparable to that found for CIP in concentrations ranging from 1.0 to 4.0 $\mu\text{g/ml}$. It required twofold-larger amounts of AZA to inhibit growth, with concentrations ranging from 2.0 to 8.0 $\mu\text{g/ml}$. *Mycobacterium avium* (ATCC 35712) was resistant to both thiopurine drugs, even at the 64.0 $\mu\text{g/ml}$ (Table 3).

In general, *M. paratuberculosis* isolates originating from Crohn's disease patients were more susceptible to thiopurine drugs than were bovine-origin isolates among tested strains. The patterns of drug susceptibility to CIP were the same regardless of isolate origin. One human isolate, UCF-7, failed to grow after 56 days of incubation in MGIT ParaTB medium

TABLE 3. Lowest concentration to inhibit^a and concentration to kill^b seven *M. paratuberculosis* strains and one *M. avium* type strain by the MGIT 960 drug susceptibility test

Drug	Concn (µg/ml) required to inhibit (kill)							
	<i>M. paratuberculosis</i> strain:							<i>M. avium</i> ATCC 35712
	JTC303	UCF-4	UCF-5	UCF-7	B213	B236	B238	
6-MP	1.0 (32.0)	2.0 (>32.0)	1.0 (>32.0)	<1.0 (8.0)	4.0 (>32.0)	4.0 (>32.0)	4.0 (>32.0)	>64.0
AZA	2.0 (>32.0)	4.0 (>32.0)	2.0 (>32.0)	1.0 (16.0)	8.0 (>32.0)	8.0 (>32.0)	16.0 (>32.0)	>64.0
AZM	1.0 (4.0)	1.0 (2.0)	0.5 (2.0)	0.5 (1.0)	1.0 (2.0)	0.5 (4.0)	>0.5 (1.0)	1.0 (16.0)
CIP	2.0 (8.0)	1.0 (4.0)	2.0 (4.0)	1.0 (4.0)	4.0 (16.0)	4.0 (16.0)	4.0 (16.0)	16.0 (64.0)

^a The lowest drug concentration producing a TTD greater than that for the 1:100 inoculum dilution control tube.

^b Signal negative by the BACTEC MGIT 960 instrument for up to 56 days postinoculation.

containing 8.0 µg/ml 6-MP or 16.0 µg/ml AZA, while the growth of the other strains was eventually detected even in the presence of 32.0 µg/ml 6-MP or AZA.

Phase III. Phase III verified the susceptibility of *M. paratuberculosis* to thiopurine drugs and showed that the effect was relatively specific for this mycobacterial species. Both 6-MP and AZA showed a stronger in vitro antimicrobial activity against 11 *M. paratuberculosis* strains than 7 *M. avium* strains; however growth inhibition patterns for thiopurine drugs against *M. avium* strains varied widely. The mean 6-MP concentrations for *M. paratuberculosis* growth inhibition ranged from <1.0 to 4.0 µg/ml, while those for *M. avium* ranged 8.0 to >128.0 µg/ml. Among *M. avium* strains, lower concentrations of the test compounds were needed to inhibit growth for isolates originating from water compared to clinical samples. The 6-MP initial inhibition range for human-origin *M. paratuberculosis* was <1.0 to 2.0 µg/ml, while for bovine-origin strains it ranged from 2.0 to 4.0 µg/ml. Again, 6-MP was found to inhibit *M. paratuberculosis* growth at one-half the concentration of AZA regardless of *M. paratuberculosis* strain origin. This finding was observed in other susceptible mycobacteria as well. No growth in MGIT ParaTB medium with 8.0 to 16.0 µg/ml of 6-MP was observed for three of the five Crohn's disease patient *M. paratuberculosis* isolates by 56 days, while growth of all *M. avium* strains was detected within 15 days at these same drug concentrations (Table 4).

The mycobacterial control *M. phlei* was as susceptible to thiopurine drugs as *M. paratuberculosis*, but *M. smegmatis* growth was completely resistant at 128 µg/ml of both thiopurine drugs. No antibacterial activity of thiopurine drugs against *E. coli* or *E. faecalis* was found, even at the highest concentration tested (128.0 µg/ml) (Table 4).

Comparison of drug actions against *M. paratuberculosis*. The drugs studied were either bacteriostatic or bactericidal or had no effect on *M. paratuberculosis*. Those with no effect, as evidenced by both plate count and MGIT 960 TTD data after either 3 or 5 days of bacterial exposure to the drugs, included the sulfonamide family (SS, SP, and 5-ASA; data not shown) and NAL (Fig. 1A).

Antimicrobial drugs AZM, CIP, and RIF were bactericidal for *M. paratuberculosis*. The minimum concentration for each drug needed to kill the organism was defined as the drug concentration producing fewer CFU than the non-drug-containing 1:100 inoculum dilution control (Fig. 1A and B). Killing concentrations for AZM and CIP by plate counting and MGIT methods were in agreement. In the presence of RIF at lower

doses, viable *M. paratuberculosis* numbers initially declined but then the organism resumed growth (Fig. 2C). Bovine- and human-origin *M. paratuberculosis* strains were similarly affected by the compounds; *M. paratuberculosis* was more sus-

TABLE 4. Lowest concentrations of two thiopurine drugs to inhibit^a and kill^b strains of *M. paratuberculosis*, *M. avium*, *M. smegmatis*, and *M. phlei*

Bacterial strain	Lowest concn of:			
	6-MP for:		AZA for:	
	Inhibition	Killing	Inhibition	Killing
<i>M. paratuberculosis</i>				
ATCC 19698	1.0	16.0	2.0	16.0
UCF-3	<1.0	8.0	2.0	32.0
UCF-4	2.0	>32.0	4.0	>32.0
UCF-5	1.0	>32.0	2.0	>32.0
UCF-7	<1.0	8.0	2.0	16.0
UCF-8	<1.0	16.0	2.0	32.0
B213	2.0	>32.0	4.0	>32.0
B236	4.0	>32.0	4.0	>32.0
B238	2.0	>32.0	4.0	>32.0
B244	4.0	>32.0	8.0	>32.0
<i>M. avium</i>				
ATCC 35712	>64.0	>64.0	>64.0	>64.0
ATCC 25291	>64.0	>64.0	>64.0	>64.0
104	>64.0	>64.0	>64.0	>64.0
JTC4862	32.0	>64.0	64.0	>64.0
JTC981	>64.0	>64.0	>64.0	>64.0
EPA3	16.0	>64.0	32.0	>64.0
WSLH1544	8.0	>64.0	32.0	>64.0
Rapid growing mycobacteria				
<i>M. phlei</i> ATCC 11758	1.0	16.0	2.0	32.0
<i>M. smegmatis</i> ATCC 14468	>128.0	>128.0	>128.0	>128.0
<i>M. smegmatis</i> mc ² 155	>128.0	>128.0	>128.0	>128.0
Nonmycobacterial controls				
<i>E. coli</i> ATCC 25922	>128.0	>128.0	>128.0	>128.0
<i>E. faecalis</i> ATCC 29212	>128.0	>128.0	>128.0	>128.0

^a The lowest drug concentration producing a TTD greater than that for the 1:100 inoculum dilution control tube.

^b Signal negative in the BACTEC MGIT 960 instrument for up to 56 days postinoculation.

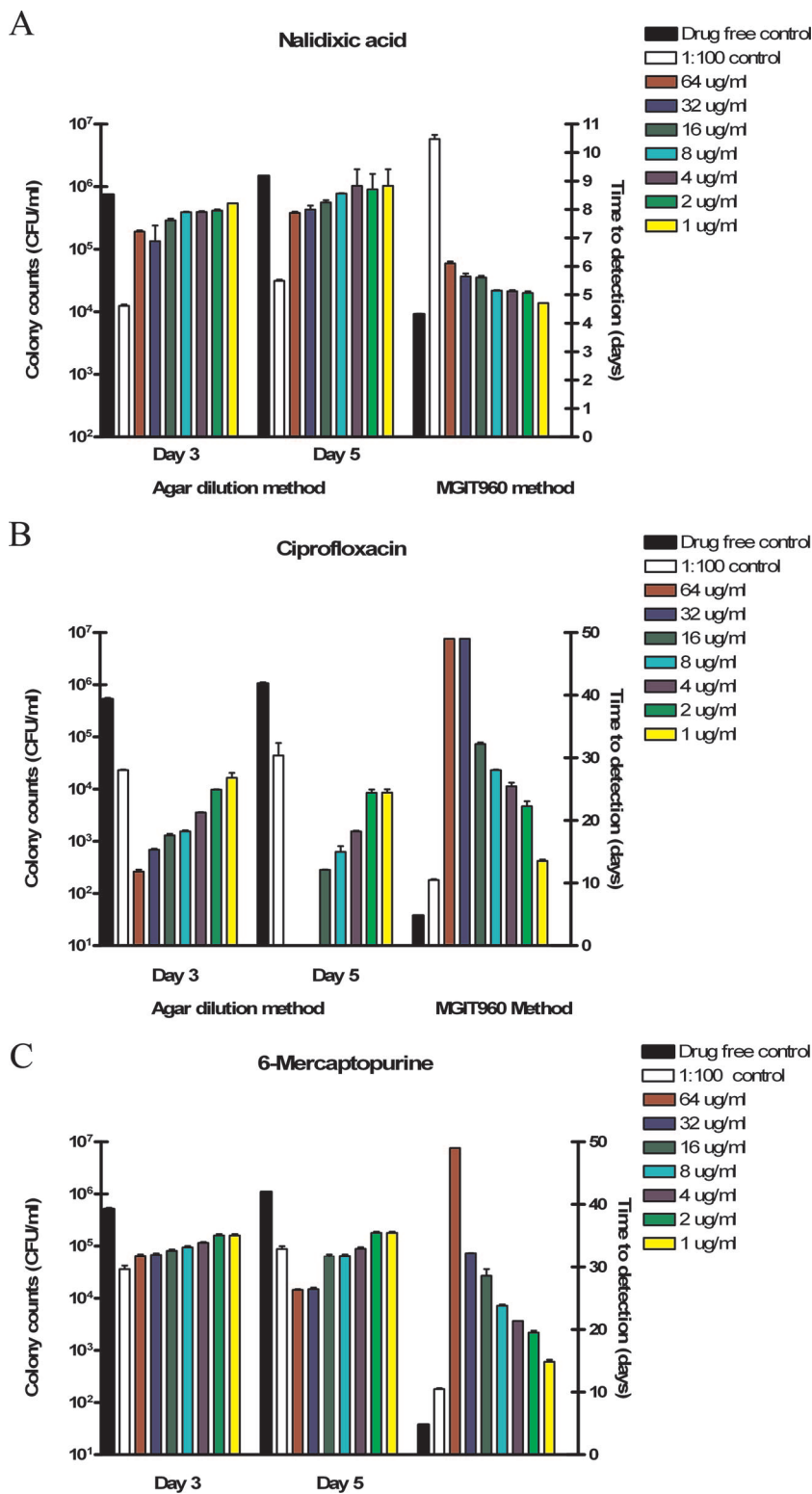


FIG. 1. Inhibition of *M. paratuberculosis* ATCC 19698 growth by exposure to NAL, CIP, and 6-MP at 1 to 64 $\mu\text{g/ml}$ for 3 or 5 days as determined by agar plate counts, and the impact of the same drug concentrations on TTD in MGIT ParaTB medium, as monitored by the BACTEC MGIT 960 instrument.

ceptible to these antibiotics than was the control *M. avium* strain tested (Table 3). The concentration of CIP required for complete *M. paratuberculosis* growth inhibition after up to 56 days of incubation in MGIT ParaTB medium for human iso-

lates was twofold lower than that for bovine isolates, i.e., 4.0 $\mu\text{g/ml}$ and 16.0 $\mu\text{g/ml}$, respectively.

Thiopurine drugs AZA and 6-MP inhibited *M. paratuberculosis* growth in a dose-dependent fashion. Plate count CFU

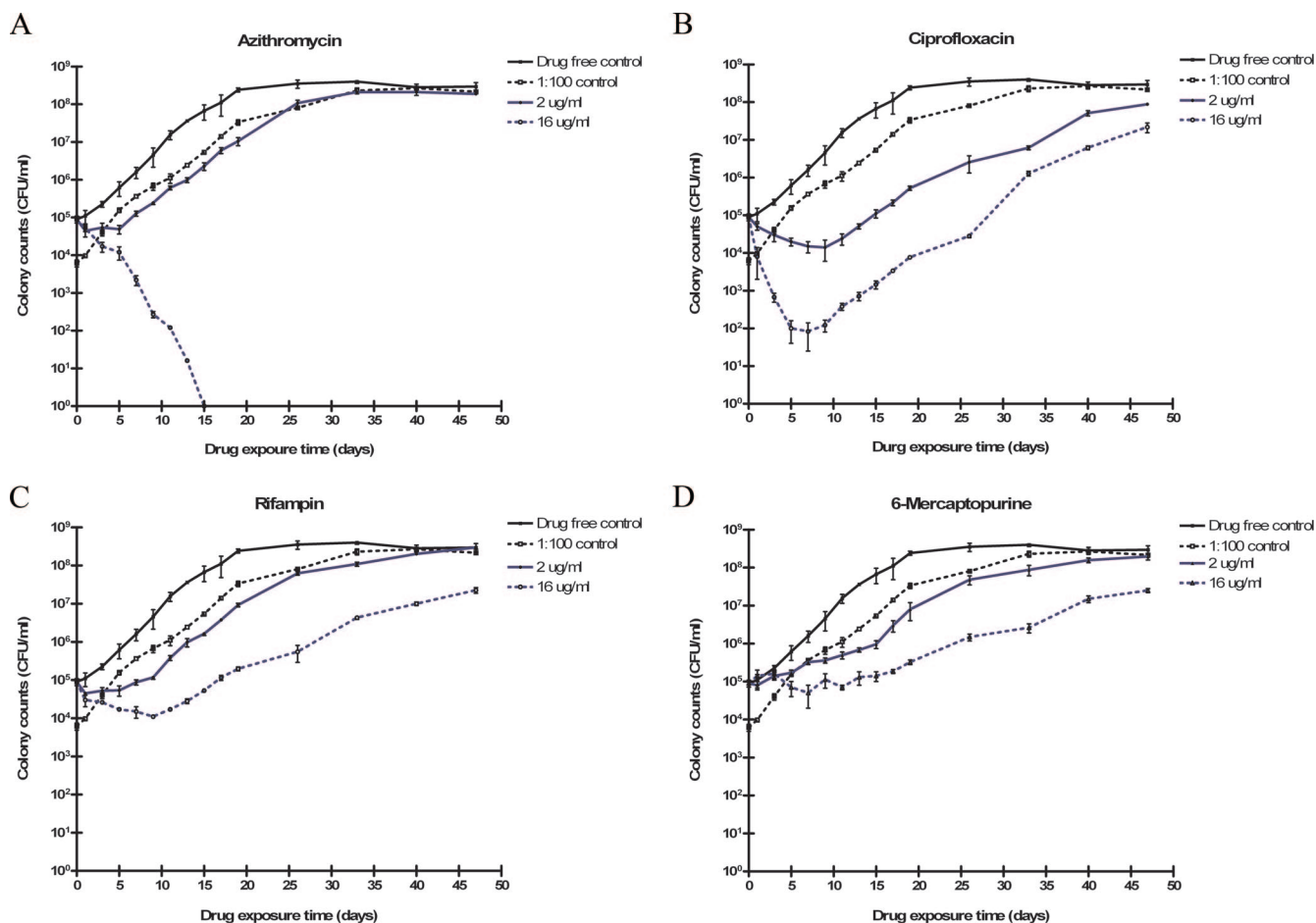


FIG. 2. Effect of AZM (A), CIP (B), RIF (C), and 6-MP (D) on the viability of *M. paratuberculosis* ATCC 19698 over time (up to 56 days), as determined by standard plate counts (CFU).

were lower than the number obtained by the MGIT 960 culture counting method but generally higher than that for the 1:100 dilution control after both 3 and 5 days of *M. paratuberculosis* exposure to the drugs (Fig. 1C). The effect of these drugs appeared more profound by the MGIT 960 drug susceptibility method, where there was continuous contact of the drug with *M. paratuberculosis* in broth, i.e., the TTD was greater than that for the 1:100 inoculum dilution control for all concentrations of drug tested (≥ 1.0 $\mu\text{g/ml}$). MGIT 960 analysis also showed a direct relationship between AZA or 6-MP concentration and suppression of *M. paratuberculosis* growth, i.e., longer TTD.

Mycobacterial species specificity for thiopurine drugs. Not all mycobacterial species were affected by thiopurine drugs to the same extent: *M. paratuberculosis* and *M. phlei* were susceptible to growth inhibition effects, but *M. avium* and *M. smegmatis* were not (Fig. 3).

Phase IV. Exposure to ≥ 2 $\mu\text{g/ml}$ 6-MP resulted in lower *M. paratuberculosis* CFU than the CFU in the 1:100 inoculum dilution control at every drug exposure time beyond 6 days. Simultaneous exposure of *M. paratuberculosis* to 6-MP and CIP at the same concentrations resulted in significantly higher CFU than use of CIP alone (Fig. 4).

DISCUSSION

Results for the antimicrobial susceptibility of *M. paratuberculosis* to standard drugs were consistent between plate counting and MGIT 960 methods (Fig. 1). When growth-suppressive effects were observed, they were drug concentration dependent. Additionally, the results were consistent with those previously reported (31, 45).

Immunosuppressive drugs in the sulfonamide family, SS, 5-AZA, and SP, had no effect on *M. paratuberculosis* growth, even at 64 $\mu\text{g/ml}$ (Table 2). NAL was tolerated by *M. paratuberculosis* up to the maximum concentration tested, 64.0 $\mu\text{g/ml}$, supporting its use in primary *M. paratuberculosis* culture media for suppression of contaminating microflora (Table 1 and Fig. 1).

AZM was bactericidal for *M. paratuberculosis* at concentrations of 4.0 to 16.0 $\mu\text{g/ml}$ (Fig. 2A). These findings are in agreement with reports of improved clinical status of Crohn's disease patients treated with macrolides (14). CIP, by contrast, killed 1 to 3 \log_{10} of *M. paratuberculosis* ATCC 19698 cells over the first 5 to 8 days, and then a seemingly drug-resistant population of cells resumed growth at rates comparable to the that for the drug-free controls (Fig. 2B). (An alternative explanation for these results is that by day 8 the CIP was no longer

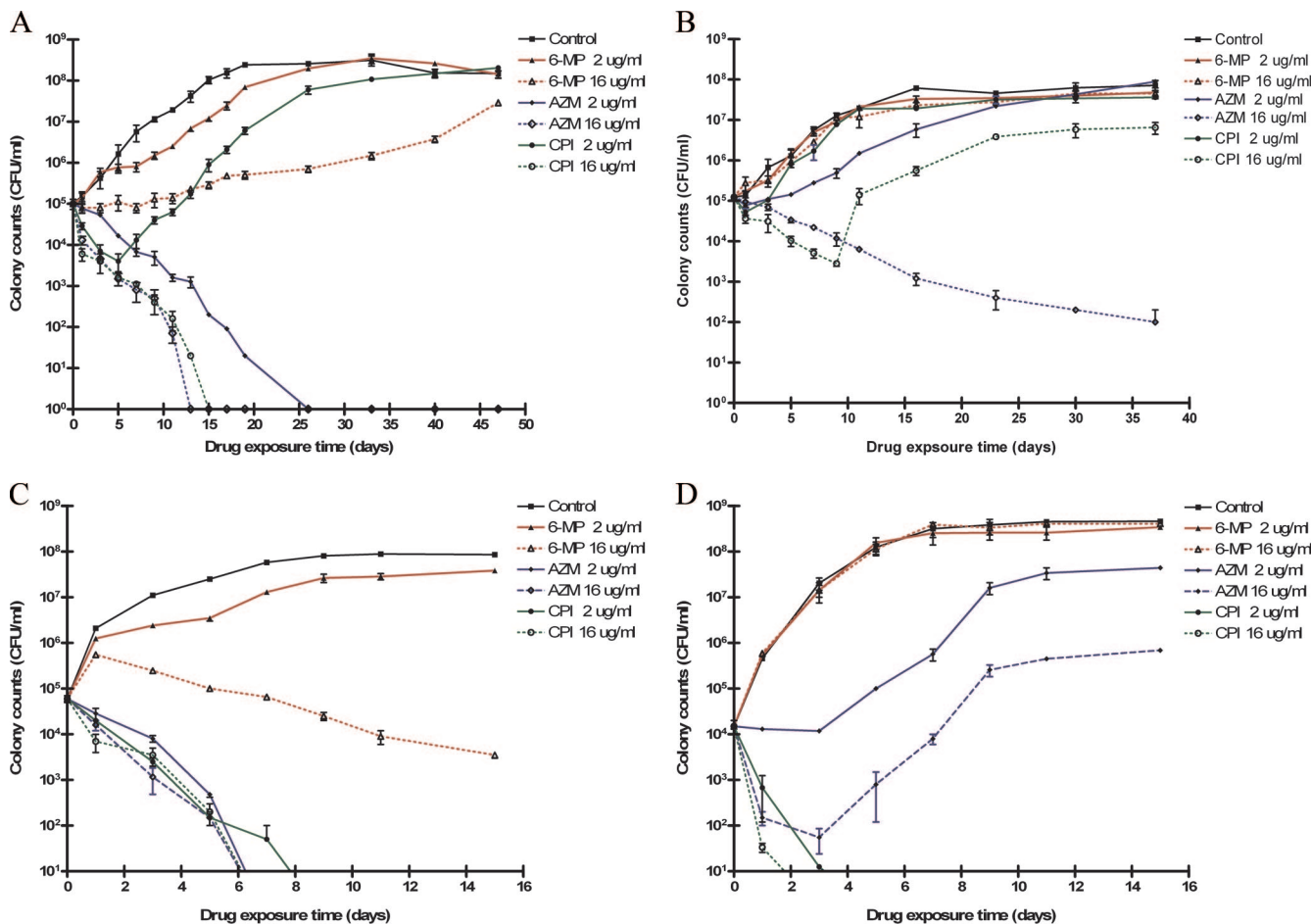


FIG. 3. Effect of AZM, CIP, and 6-MP on the viability of *M. paratuberculosis* strain UCF-5 (A), *M. avium* ATCC 35712 (B), *M. phlei* ATCC 11758 (C), and *M. smegmatis* mc²155 (D) over time (up to 56 days), as determined by agar plate counts (CFU).

active, and future studies should evaluate residual drug activity.) Human-origin strains were more susceptible to CIP, with complete killing of 10⁵ CFU *M. paratuberculosis* in 12 to 26 days (Fig. 3A). RIF produced an *M. paratuberculosis* growth

pattern similar to that produced by CIP, i.e., transitory decline in CFU followed by regrowth (Fig. 2B and C).

AZA drugs killed *M. paratuberculosis*. The thiopurine drug 6-MP suppressed its growth more than did AZA at the same

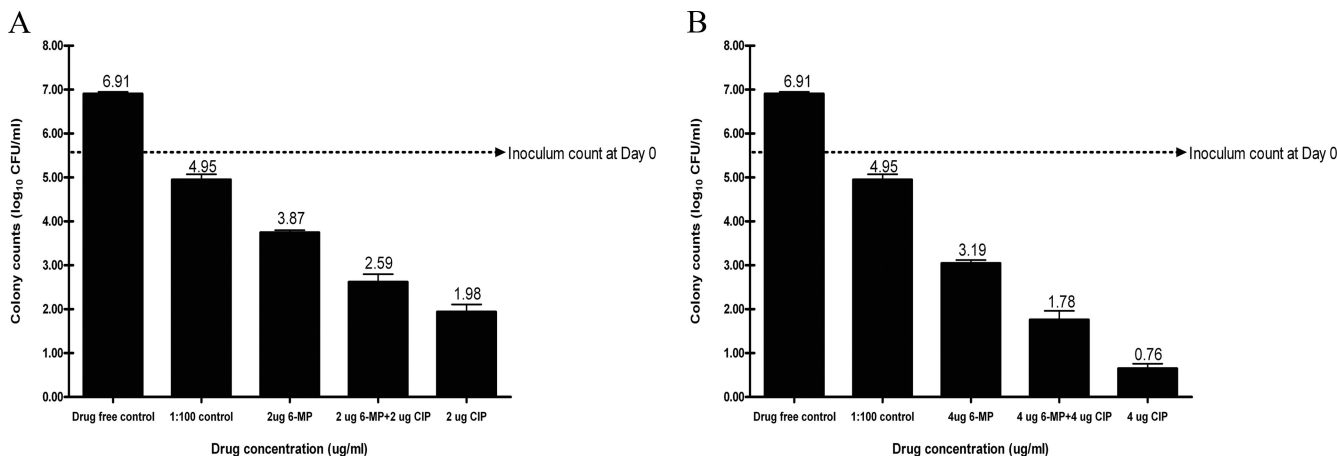


FIG. 4. Effect of CIP, 6-MP, and the combination of the two drugs both at 2 μ g/ml (A) and 4 μ g/ml (B) on the viability of *M. paratuberculosis* strain UCF-7 after 12 days of drug exposure as determined by MGIT 960 counting methods (38).

concentrations ($\mu\text{g/ml}$). If MGIT drug susceptibility standards for *M. tuberculosis* were used to interpret the data for 6-MP versus *M. paratuberculosis*, growth was suppressed at ≥ 2.0 $\mu\text{g/ml}$ (potentially the MIC) compared with the 1:100 drug-free growth control (Fig. 2D). However, growth of *M. paratuberculosis* was not completely stopped, even at 16 $\mu\text{g/ml}$ 6-MP, but was simply slowed in comparison to that of drug-free controls. Human-origin (Crohn's disease patient) isolates of *M. paratuberculosis* tended to be more susceptible to 6-MP than were bovine-origin isolates (Table 4). These findings are comparable to those reported by Greenstein et al. using the BACTEC 460 system for *M. paratuberculosis* in vitro susceptibility studies (13, 45).

The growth-suppressive effects of 6-MP differed by mycobacterial species. These data suggest a possible mycobacterial-species-specific mechanism by which 6-MP interferes with replication or metabolism.

The antimycobacterial effect of thiopurine drugs is a novel and unexpected observation. The implications of the present study for the possible etiologic role of *M. paratuberculosis* in Crohn's disease and approaches to Crohn's disease therapy are important. Some investigators describe the failure of *M. paratuberculosis* to multiply in the face of immunosuppressive therapy with thiopurine drugs as evidence that Crohn's disease cannot be caused by *M. paratuberculosis* (35). Perhaps this pathogen, found in resected bowel tissue and peripheral blood leukocytes of some Crohn's disease patients (6, 25, 36), is held in check by thiopurine therapy since it has both immunosuppressive and anti-*M. paratuberculosis* activity.

These data also offer another perspective on data from clinical trials with Crohn's disease patients using antimycobacterial drugs. Since patients in the "control" group are maintained on standard therapy (which commonly employs thiopurine drugs), both the treatment group (antimycobacterial drugs) and control group (no antimycobacterial drugs but continued thiopurine drugs) are exposed to compounds with anti-*M. paratuberculosis* activity. If *M. paratuberculosis* is integral to Crohn's disease, then the opportunity to observe clear-cut therapeutic differences between the treatment and control groups in these trials is limited.

Antimycobacterial and thiopurine drugs used in concert may produce an interactive effect. The apparently bacteriostatic effects of 6-MP on *M. paratuberculosis* rendered the organism less susceptible to the bactericidal effects of CIP. This further complicates interpretation of many of the prior clinical trials with Crohn's disease patients that employed antimicrobials in addition to immunosuppressive drugs. Given the potential side effects of these medications, pursuit of therapeutic trials with patients in the absence of sound in vitro data is both premature and inappropriate.

A major challenge for antimicrobial susceptibility studies of *M. paratuberculosis* is its long generation time, i.e., roughly 2 days (24), and uncertainty about the stability of the test drugs in MGIT ParaTB medium at 37°C over the course of incubation. Without accepted standards for *M. paratuberculosis* antimicrobial susceptibility testing, it was vital that multiple methods and controls be employed and that results be descriptive and interpreted in relative rather than absolute terms such as "susceptible" or "resistant." Multiple mycobacterial species as well as nonmycobacterial species were needed as antibiotic susceptibility quality control standards. We also assessed

whether drugs had an inhibitory or lethal effect on target organisms by subculture to drug-free media. More-extensive in vitro drug susceptibility trials with *M. paratuberculosis* are required to establish which drugs are most efficacious and which drugs, when used in combination, have a modulated effect on *M. paratuberculosis*. While the results of this research are provocative, expanded studies should include an assessment of the stability and activity of antimicrobial drugs in MGIT ParaTB medium at 37°C over the extended incubation period required for *M. paratuberculosis* drug susceptibility testing.

This work is hypothesis generating, not definitive. Methodological issues, in particular, that the mycobacteria were tested in an extracellular location, and the limited number of strains of each mycobacterial species tested are among just some of the caveats regarding extension of these findings to the clinical situation. However, this work highlights the complexity of studying *M. paratuberculosis* interactions with antibiotics and the possibility that anti-inflammatory drugs may exert antibacterial effects directly on this organism and/or have negative interactions with conventional antimicrobial drugs. These caveats argue against simple adoption of drug susceptibility testing methods used for *M. tuberculosis* or *M. avium* and for more-comprehensive characterization of the bactericidal or growth-inhibitory effects of antimicrobial and anti-inflammatory drugs alone and in combination on *M. paratuberculosis*.

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