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# Long-Term Shifts in Patterns of Antibiotic Resistance in Enteric Bacteria

TARA HOUNDT AND HOWARD OCHMAN*

*Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona 85721*

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**Several mechanisms are responsible for the ability of microorganisms to tolerate antibiotics, and the incidence of resistance to these compounds within bacterial species has increased since the commercial use of antibiotics became widespread. To establish the extent of and changes in the diversity of antibiotic resistance patterns in natural populations, we determined the MICs of five antibiotics for collections of enteric bacteria isolated from diverse hosts and geographic locations and during periods before and after commercial application of antibiotics began. All of the pre-antibiotic era strains were susceptible to high levels of these antibiotics, whereas 20% of strains from contemporary populations of *Escherichia coli* and *Salmonella enterica* displayed high-level resistance to at least one of the antibiotics. In addition to the increase in the frequency of high-level resistance, background levels, conferred by genes providing nonspecific low-level resistance to multiple antibiotics, were significantly higher among contemporary strains. Changes in the incidence and levels of antibiotic resistance are not confined to particular segments of the bacterial population and reflect responses to the increased exposure of bacteria to antimicrobial compounds over the past several decades.**

How much diversity exists in the antibiotic resistance patterns within natural populations of enteric bacteria, and how have the levels of resistance changed since the use of antibiotics became widespread? Increased introduction of antimicrobial agents into the environment via medical therapy, agriculture, and animal husbandry (5, 8) has resulted in new selective pressures on bacterial populations. This has exacerbated the problem of controlling microbes in a disease setting and has caused a resurgence of bacterial diseases worldwide due to the acquisition and transfer of virulence factors and antibiotic resistance genes (17, 30). These problems are further compounded by the persistence of resistance determinants in bacterial genomes over hundreds of generations, even in the absence of antibiotics as selective agents (14).

There are two general categories of antibiotic resistance traits displayed by microorganisms: (i) those that allow microorganisms to withstand relatively high levels of a specific antimicrobial agent, which are conferred by mutations in genes responsible for antibiotic uptake or binding sites, as well as those gained by acquisition of genes on mobile elements (6, 7, 18); and (ii) those provided by genes conferring nonspecific low-level resistance to multiple antibiotics, such as the multiple antibiotic resistance (*mar*) locus (1, 9, 11). Although dissemination of resistance genes by mobile elements provides a rapid response to antibiotic challenge, increases in background levels of resistance might also be expected in natural populations in environments augmented with antibiotics.

To examine the ways in which increased exposure to antibiotics over the past several decades has changed the patterns and levels of resistance in natural populations of bacteria, we compared MICs for enteric strains isolated before the use of antibiotics became widespread to MICs for contemporary populations that vary in exposure to antibiotics due to their host environments. Among pre-antibiotic era strains the prevalence of genes conferring very high levels of resistance is known to be low due to the lack of strong selective pressures (13, 16). Similarly, the background levels of resistance in these strains might also be expected to be lower than those of strains isolated since the use of antibiotics became widespread even in contemporary strains not collected as a direct result of infection and antibiotic treatment.

Host ecology and environment also factor into the patterns of antibiotic resistance in natural populations. For example, strains of *Salmonella* are likely to have experienced different selective pressures for resistance than *Escherichia coli* strains have experienced because they reside primarily in nonmammalian hosts and are far less likely to have encountered most commercial antibiotics in their natural environments. However, such organisms have been exposed to naturally occurring antimicrobial agents, such as the small-polypeptide defensins, that are distributed broadly in mammalian and nonmammalian hosts. Hence, the patterns of resistance of enteric bacteria to these antimicrobial agents likely differ from the patterns produced as a result of challenge by commercially applied antibiotics. By determining the MICs of several antibiotics for bacterial collections representing different temporal and host populations, we determined that the environmental occurrence of antibiotics is reflected in the resistance patterns displayed by enteric bacteria and that the incidence and levels of antibiotic resistance in bacterial strains have changed in response to the increased application of antibiotics.

## MATERIALS AND METHODS

**Bacterial strains.** The collections of natural populations of enteric bacteria chosen for this study were as follows. (Full details concerning the sources, strain designations, and years of isolation are given in the references cited.) (i) The ECOR collection (22) of *E. coli* strains contains 72 strains isolated from natural populations between 1972 and 1982. The strains were derived from numerous host types, including healthy humans from New York, Iowa, and Sweden, humans with urinary tract infections, and domesticated, zoo-kept, and wild-caught mammals. Strains in this collection were originally selected to encompass a broad range of hosts and geographic locations and to represent the genotypic diversity within this species, as determined by multilocus enzyme electrophoresis (12, 25). The MICs of several antibiotics and the presence of integrons near antibiotic resistance cassettes have been surveyed with this collection (18). (ii) The SARC collection of *Salmonella* strains contains 20 strains isolated between 1958 and 1987 representing the major subspecific groups of *Salmonella enterica* (groups I, II, IIIa, IIIb, IV, VI, and VII) and *Salmonella bongori* (group V), as defined by

* Corresponding author. Mailing address: Department of Ecology and Evolutionary Biology, 233 Life Sciences South, University of Arizona, Tucson, AZ 85721. Phone: (520) 626-8355. Fax: (520) 621-3709. E-mail: hochman@email.arizona.edu.

genetic and phenotypic characteristics (3). Whereas members of these species normally circulate in nonmammalian hosts, as represented by strains isolated from a snake, a frog, and a bird, most of the SARC strains were recovered from humans with opportunistic infections. (iii) Bacteria from the pre-antibiotic era were represented by *E. coli* strains amassed by E.D.G. Murray (13). These strains were recovered primarily from humans with infections between 1885 and 1941 and were collected over a wide geographic range. The antibiotic resistance profiles of these and other natural strains of *E. coli* have also been reported by Routman et al. (24).

**Antibiotic resistance profiles.** The MICs of five antibiotics (ampicillin, chloramphenicol, kanamycin, tetracycline, and protamine) were determined by the gradient plate method of Szybalski and Bryson (29). Most of the gradient plates contained 1.5% Luria-Bertani (LB) agar; the exceptions were the plates containing protamine, which contained 1.1% LB agar. When plates were prepared, LB agar containing the appropriate antibiotic was poured as the bottom layer because this method produced more accurate and reproducible results. Plates were incubated at room temperature overnight prior to use to ensure proper diffusion of the antibiotic. Streaked plates were incubated overnight at 37°C, and the MIC for a strain was obtained by averaging the results for two to four replicates and then rounding to the nearest whole number for all of the antibiotics except protamine. Statistical tests were performed in Statview 1.0, and the differences in mean values were compared by Mann-Whitney U tests.

## RESULTS

MIC profiles for strains from each of the three collections are shown in Tables 1 through 3. For each of the commercially administered antibiotics (ampicillin, chloramphenicol, kanamycin, and tetracycline), the distributions of MICs were essentially bimodal, with the two modes corresponding to high-level and background resistance. (The exact distributions were different for different antibiotics, but in general, high-level resistance means resistance to concentrations of >50 μg/ml, whereas background levels were less than 10 to 20 μg/ml depending on the antibiotic.)

**High-level resistance to antibiotics.** High-level resistance to commercially applied antibiotics was more common among contemporary strains than among strains from the pre-antibiotic era. (In fact, Murray strain 125, the single pre-antibiotic era strain displaying high-level resistance, was probably a contaminant because its MIC profile did not correspond to that reported previously [13].) Approximately 20% of strains in both the ECOR and SARC collections displayed high-level resistance to at least one antibiotic, and less than 5% were resistant to two. The overall frequencies of resistant strains were similar to those reported previously for these and other antibiotics surveyed with the ECOR collection (18). For the majority of antibiotics, the incidences of high-level resistance were similar for strains from human and nonhuman hosts; however, the rare cases of high-level resistance to kanamycin and high-level resistance to chloramphenicol were detected only among strains from nonhuman hosts. Within the ECOR collection, there was a prevalence of high-level resistance to tetracycline (14% of the strains were resistant to concentrations of >50 μg/ml), whereas 20% of the strains in the SARC collection showed high-level resistance to ampicillin.

The ECOR and SARC collections each contain several pairs of strains known to be closely related based on electrophoretically detectable variation in chromosomally encoded enzyme loci. In two of these cases, strains ECOR 18 and ECOR 19 and strains ECOR 38 and ECOR 39, the pairs of closely related strains displayed high levels of resistance to tetracycline. In contrast, only ECOR 10, although it is genetically identical to ECOR 11 as determined by multilocus enzyme electrophoresis, had a high level of resistance to ampicillin, presumably due to very recent acquisition of a resistance gene. Among the salmonellae, SARC 11 and SARC 12, both of which are classified as *S. bongori*, had similarly high levels of resistance to ampicillin, despite the fact that they were isolated 4 years apart from different host species.

**Analysis of background resistance patterns.** The mean MICs of kanamycin, ampicillin, tetracycline, chloramphenicol, and protamine for each of the collections are given in Table 4. Even when the strains displaying high-level resistance to a particular antibiotic were eliminated from the comparisons, the average MIC of each antibiotic was significantly higher for the ECOR collection than for the Murray collection. Similarly, the mean MICs for the ECOR collection were significantly higher than those for the SARC collection for all antibiotics except protamine (to which the salmonellae were significantly more tolerant).

The ECOR collection has been subdivided into five phylogenetic subgroups (subgroups A, B1, B2, D, and E) (12), and some of these subspecific groups differed significantly in their levels of background resistance to particular antibiotics. For example, the mean MICs of tetracycline and chloramphenicol were significantly lower for subgroup B2 organisms than for subgroup B1 organisms, although subgroup B2 strains had the highest average levels of resistance to ampicillin. Despite encompassing a much larger range of genetic diversity, *Salmonella* strains were comparatively much less variable in their levels of background resistance than *E. coli* strains were.

## DISCUSSION

Expanded application of antibiotics has caused an increase in the incidence of resistance to these antimicrobial compounds, even within bacterial species that are not directly subject to antibiotic control. Numerous genes conferring resistance to antibiotics are presently circulating in bacterial populations, and such factors were not as prevalent prior to the selective pressures produced by the increased use of antibiotics (6, 13). Therefore, it is not surprising that contemporary strains of both *E. coli* and *Salmonella* frequently display resistance to high levels of commercially administered antibiotics, and such cases have been documented repeatedly with these and other bacteria (10).

However, aside from the anticipated increase in the incidence of high-level antibiotic resistance in contemporary populations, we also detected a change in the background levels of resistance. Even when all cases of high-level resistance were excluded from our analyses, the recently isolated strains of *E. coli* displayed significantly higher MICs than the pre-antibiotic era Murray strains displayed. This shift was not an artifact of sampling two genetically distinct or restricted subsets of the *E. coli* population; strains from the Murray collection represent four of the five phylogenetic groups of *E. coli* and encompass almost the same range of genotypic variation and the same levels of genic diversity ($H$) as the ECOR collection strains encompass ($H_{ECOR}$ is 0.34 and $H_{Murray}$ is 0.27 based on electrophoretic variation at 35 and 10 enzyme loci, respectively). Hence, changes in background levels of antibiotic resistance affect the species as a whole and seem likely to be a response to increased exposure to the compounds over the past several decades. In addition, given that the increase was observed for all of the antibiotics tested, it was presumably caused by refinement of loci conferring nonspecific low-level resistance to multiple antibiotics.

If environmental exposure to antibiotics can elevate levels of the antibiotic resistance, then host populations should directly influence antibiotic resistance profiles. Consistent with this reasoning, the background levels of antibiotic resistance for commercially applied antibiotics are higher in *E. coli* strains, which are commensals in humans and domesticated mammals, than in *Salmonella* strains, which circulate principally in nonmammalian populations. In addition, it is noteworthy that *Sal-*

TABLE 1. MICs for *E. coli* strains constituing the ECOR collection

| ECOR no. | Host[a] | MIC of: | | | | |
|---|---|---|---|---|---|---|
| | | Kanamycin (μg/ml) | Ampicillin (μg/ml) | Tetracycline (μg/ml) | Chloramphenicol (μg/ml) | Protamine (mg/ml) |
| 1 | H | 13 | 8 | 4 | 5 | 1.4 |
| 2 | H | 32 | 4 | 2 | 3 | 1.5 |
| 3 | NH | 15 | 4 | 107 | 4 | 1.3 |
| 4 | H | 13 | 13 | 150 | 8 | 1.1 |
| 5 | H | 10 | 7 | 2 | 4 | 1.1 |
| 6 | H | 15 | 11 | 54 | 4 | 1.1 |
| 7 | NH | 13 | 7 | 5 | 4 | 2.5 |
| 8 | H | 3 | 14 | 2 | 2 | 1.6 |
| 9 | H | 12 | 2 | 1 | 1 | 1.5 |
| 10 | H | 14 | >200 | 2 | 6 | 1.6 |
| 11 | H[b] | 17 | 3 | 2 | 2 | 1.5 |
| 12 | H | 13 | 6 | 3 | 5 | 1.6 |
| 13 | H | 15 | 7 | 4 | 7 | 1.0 |
| 14 | H[b] | 8 | 6 | 3 | 5 | 1.6 |
| 15 | H | 15 | 7 | 4 | 4 | 1.1 |
| 16 | NH | 11 | 4 | 39 | 6 | 1.8 |
| 17 | NH | 13 | 2 | 150 | 4 | 1.8 |
| 18 | NH | 9 | 3 | 122 | 6 | 1.6 |
| 19 | NH | 25 | 4 | 156 | 6 | 1.6 |
| 20 | NH | 9 | 4 | 2 | 4 | 1.6 |
| 21 | NH | 20 | 14 | 3 | 4 | 1.8 |
| 22 | NH | 12 | 14 | 3 | 7 | 1.2 |
| 23 | NH | 10 | 6 | 4 | 7 | 1.7 |
| 24 | H | 21 | 2 | 3 | 1 | 1.6 |
| 25 | NH | 14 | 15 | 3 | 4 | 1.6 |
| 26 | H | 14 | 10 | 3 | 7 | 2.5 |
| 27 | NH | 10 | 8 | 5 | 7 | 2.0 |
| 28 | H | 7 | 11 | 5 | 7 | 1.6 |
| 29 | NH | 7 | 6 | 5 | 3 | 1.5 |
| 30 | NH | 11 | 6 | >200 | 4 | 2.0 |
| 31 | NH | >150 | >200 | 4 | 4 | 1.6 |
| 32 | NH | 8 | 6 | 4 | 6 | 1.7 |
| 33 | NH | 13 | 7 | 4 | 6 | 2.0 |
| 34 | NH | 9 | 6 | 4 | 7 | 1.8 |
| 35 | H | 7 | 6 | 4 | 3 | 2.5 |
| 36 | H | 10 | 4 | 10 | 3 | 2.0 |
| 37 | NH | >150 | 8 | 3 | >200 | 2.0 |
| 38 | H | 7 | 10 | 150 | 4 | 1.9 |
| 39 | H | 3 | 15 | 145 | 2 | 1.9 |
| 40 | H[b] | 3 | 6 | 3 | 3 | 2.1 |
| 41 | H | 20 | 9 | 4 | 4 | 2.0 |
| 42 | H | 14 | 28 | 8 | 6 | 0.7 |
| 43 | H | 10 | 5 | 3 | 6 | 0.5 |
| 44 | NH | 9 | 10 | 4 | 11 | 1.2 |
| 45 | NH | 8 | 7 | 3 | 7 | 0.7 |
| 46 | NH | 8 | 5 | 63 | 3 | 1.1 |
| 47 | NH | 15 | 24 | 3 | 8 | 0.8 |
| 48 | H[b] | 18 | 11 | 28 | 6 | 1.0 |
| 49 | H | 15 | 30 | 1 | 5 | 1.9 |
| 50 | H[b] | 13 | 8 | 2 | 6 | 1.1 |
| 51 | H | 18 | 5 | 2 | 3 | 1.8 |
| 52 | NH | 4 | 14 | 2 | 6 | 1.5 |
| 53 | H | 18 | 14 | 2 | 6 | 1.5 |
| 54 | H | 5 | 20 | 3 | 4 | 1.5 |
| 55 | H | 4 | 6 | 4 | 5 | 1.6 |
| 56 | H[b] | 13 | 11 | 4 | 5 | 1.1 |
| 57 | NH | 13 | 9 | 3 | 5 | 2.5 |
| 58 | NH | 20 | 12 | 4 | 6 | 2.5 |
| 59 | H | 22 | 16 | 3 | 4 | 2.0 |
| 60 | H[b] | 14 | 8 | 3 | 5 | 1.5 |
| 61 | H | 14 | 6 | 2 | 4 | 2.0 |
| 62 | H[b] | 19 | 10 | 2 | 3 | 1.8 |
| 63 | H | 9 | 7 | 1 | 2 | 2.2 |
| 64 | H[b] | 17 | 13 | 2 | 3 | 1.8 |
| 65 | NH | 29 | 12 | 2 | 3 | 1.4 |
| 66 | NH | 23 | 19 | 4 | 4 | 1.7 |
| 67 | NH | 13 | 18 | 4 | 5 | 1.5 |
| 68 | NH | 22 | 2 | 2 | 4 | 1.8 |
| 69 | NH | 10 | 3 | 3 | 4 | 1.4 |
| 70 | NH | 20 | 4 | 4 | 4 | 1.8 |
| 71 | H[b] | 19 | 22 | 4 | 10 | 1.8 |
| 72 | H[b] | 28 | 5 | 3 | 3 | 1.8 |

[a] NH, nonhuman animals; H, humans.
[b] Strain isolated from the urine of a woman with a urinary tract infection.

TABLE 2. MICs for *E. coli* strains from the pre-antibiotic era

| Murray no. | MIC of: | | | | |
|---|---|---|---|---|---|
| | Kanamycin (μg/ml) | Ampicillin (μg/ml) | Tetracycline (μg/ml) | Chloramphenicol (μg/ml) | Protamine (mg/ml) |
| 20 | 9 | 6 | 2 | 3 | 1.2 |
| 125[a] | 7 | >200 | 2 | 15 | >3.0 |
| 127 | 5 | 7 | 2 | 3 | 0.9 |
| 142 | 8 | 5 | 2 | 1 | 0 |
| 163 | 3 | 6 | 1 | 1 | 0.8 |
| 177 | 8 | 2 | 1 | 3 | 0.9 |
| 228 | 2 | 2 | 0 | 0 | 0.8 |
| 256 | 8 | 1 | 1 | 0 | 0.8 |
| 265 | 9 | 4 | 1 | 4 | 0.8 |
| 291 | 6 | 1 | 2 | 2 | 0.7 |
| 292 | 6 | 3 | 1 | 3 | 1.0 |
| 297 | 6 | 1 | 1 | 1 | 0.8 |
| 331 | 16 | 7 | 0 | 2 | 0.8 |
| 389 | 1 | 2 | 1 | 1 | 1.2 |
| 401 | 7 | 1 | 1 | 2 | 0.8 |
| 427a | 31 | 2 | 0 | 1 | 0.8 |
| 427b | 30 | 3 | 0 | 1 | 0.8 |
| 429 | 7 | 1 | 1 | 2 | 0.8 |
| 609 | 8 | 5 | 2 | 5 | 1.1 |
| 611a | 38 | 5 | 2 | 6 | 0.9 |
| 613 | 4 | 7 | 2 | 4 | 0.9 |
| 614 | 13 | 0 | 1 | 0 | 0 |
| 615 | 9 | 3 | 2 | 4 | 0.8 |
| 616 | 13 | 7 | 3 | 6 | 1.1 |
| 617 | 3 | 1 | 3 | 4 | 0.8 |
| 618 | 8 | 8 | 2 | 5 | 1.2 |
| 619 | 7 | 6 | 2 | 4 | 0.9 |
| 633 | 9 | 6 | 3 | 5 | 0.8 |
| 634 | 5 | 12 | 3 | 6 | 0.8 |
| 663 | 7 | 4 | 2 | 4 | 0.8 |
| 679 | 0 | 1 | 0 | 4 | 0.6 |
| 682 | 0 | 7 | 2 | 3 | 1.2 |

[a] Strain eliminated from the analysis (see text).

*monella* strains are, on average, more resistant to protamine, a small antimicrobial peptide whose analogs have been recovered from a wide range of vertebrate hosts. However, within *E. coli*, there is not a significant difference in the antibiotic resistance profiles of strains isolated from human and nonhuman hosts; however, strains of this species are not typically restricted to particular host species or geographic locations (23, 24, 26).

Bacteria have a wide array of mechanisms to protect the cell from destruction by an antimicrobial agent: (i) the antibiotic molecule can be modified or sequestered by genetically encoded enzymes or cellular proteins (2, 15, 27); (ii) bacteria employ active efflux mechanisms and/or permeability barriers to certain compounds (20); and (iii) the specific target of the antibiotic may be modified to make it insensitive to the drug (28). The sporadic occurrence of antibiotic resistance determinants among enteric bacteria suggests that the very high levels of resistance to certain antibiotics are due to a combination of these strategies and are transmitted by mobile genetic elements. For example, high-level resistance to tetracycline and high-level resistance to β-lactams are each known to be conferred by at least two mechanisms, which could account for their prevalence in the ECOR and SARC collections, respectively.

Despite the broad distribution of high-level antibiotic resistance in contemporary populations of *E. coli* and *Salmonella*, early characterizations of the plasmid contents of pre-antibi-

TABLE 3. MICs for *Salmonella* strains constituting the SARC collection

| SARC no.[a] | Host[b] | MIC of: | | | | |
|---|---|---|---|---|---|---|
| | | Kanamycin (μg/ml) | Ampicillin (μg/ml) | Tetracycline (μg/ml) | Chloramphenicol (μg/ml) | Protamine (mg/ml) |
| (s53) | | 4 | 4 | 2 | 3 | 1.5 |
| (s1280) | | 7 | 4 | 3 | 6 | 1.6 |
| (s1518) | | >50 | >50 | 10 | 5 | 1.6 |
| (s2962) | | 4 | 2 | 2 | 2 | 1.4 |
| 1 | H | 9 | 6 | 3 | 6 | 1.7 |
| 2 | | 8 | 2 | 1 | 3 | 1.5 |
| 3 | H | 5 | 4 | 2 | 4 | 2.6 |
| 4 | | 9 | 3 | 1 | 3 | 2.7 |
| 5 | NH | 11 | 4 | 3 | 3 | 2.3 |
| 6 | H | 10 | 3 | 2 | 3 | 2.4 |
| 7 | H | 16 | 4 | 3 | 5 | 2.9 |
| 8 | H | 13 | 1 | 1 | 2 | 2.5 |
| 9 | NH | 5 | 8 | 1 | 2 | 1.7 |
| 10 | H | 36 | 6 | 8 | 2 | 2.5 |
| 11 | NH | 6 | >50 | 2 | 5 | 2.1 |
| 12 | NH | 7 | >50 | 2 | 4 | 1.8 |
| 13 | | 3 | 2 | 2 | 2 | 2.4 |
| 14 | | 6 | 4 | 2 | 2 | 3.0 |
| 15 | | 5 | >50 | 2 | 4 | 3.0 |
| 16 | H | 7 | 25 | 1 | 4 | 3.0 |

[a] Four subgroup I strains from this collection have not been assigned SARC numbers. The original designations for these strains are given in parentheses.
[b] NH, nonhuman animals; H, humans.

otic era strains provided no evidence of transferable antibiotic resistance determinants (13, 16). Although salmonellae do not habitually reside in hosts treated with antibiotics and are likely to have experienced different selective pressures for resistance than *E. coli* strains have experienced, R plasmids and other genetic elements conferring antibiotic resistance can be efficiently maintained and disseminated within this species by conjugation, transformation, and transduction (4, 19, 21). Our future work will be directed towards identifying the specific genes or alleles that are responsible for the differences in the antibiotic resistance profiles observed within and among these organisms and to examine the MICs of additional antibiotics in other natural populations of enteric bacteria.

Application of antibiotics over the past 50 years has resulted in an unremitting increase in the numbers of commensal and pathogenic bacteria that are resistant to antimicrobial compounds. We have shown that aside from the high frequency of resistance to a particular antibiotic, contemporary populations of enteric bacteria display elevated tolerance in their nonspecific responses to several antibiotics. Although the increases in the background levels of resistance do not threaten control of these organisms, the results show that bacteria, even those not regularly or directly subjected to antibiotic challenge, have changed in response to increases in the application of antibiotics over the past several decades.

TABLE 4. Comparisons of MICs for enteric strain collections

| Collection | MIC (mean ± SD) | | | | |
|---|---|---|---|---|---|
| | Kanamycin (μg/ml) | Ampicillin (μg/ml) | Tetracycline (μg/ml) | Chloramphenicol (μg/ml) | Protamine (mg/ml) |
| ECOR | 13.5 ± 6.2 | 9.3 ± 6.0 | 4.3 ± 5.7 | 4.8 ± 1.9 | 1.62 ± 0.43 |
| Murray | 9.1 ± 8.7 | 4.1 ± 2.8 | 1.4 ± 0.8 | 2.9 ± 1.9 | 0.83 ± 0.27 |
| SARC | 7.6 ± 3.3 | 3.5 ± 1.8 | 1.9 ± 0.6 | 3.7 ± 1.3 | 2.21 ± 0.57 |

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