Fungal Transformation of an Antimicrobial Fluoroquinolone Drug During Growth on Poultry Litter Materials

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Primary Audience: Researchers, Veterinarians, Public Health Officials

SUMMARY

The ability of a nonpathogenic fungus, *Pestalotiopsis guepini*, to metabolize fluoroquinolone antimicrobial agents during growth on poultry litter materials was investigated. Sterilized rice hulls, ground corncobs, and pine shavings in glass jars covered with foil were moistened with sterile water and inoculated with *P. guepini*. The litter materials then were dosed with norfloxacin and incubated for 20 d. In rice-hull cultures, *P. guepini* produced 4 metabolites: 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinolone-3-carboxylic acid, *N*-formylnorfloxacin, *N*-acetylnorfloxacin, and desethylene-*N*-acetylnorfloxacin. In corncob cultures, the fungus produced *N*-formylnorfloxacin and *N*-acetylnorfloxacin. In pine-shavings cultures, there was little growth of the fungus and no metabolism of norfloxacin. The results suggest that fungi that grow on poultry litter may degrade residues of antimicrobial drugs.

Key words: fluoroquinolone, norfloxacin, poultry litter

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DESCRIPTION OF PROBLEM

The use of antimicrobial agents in the poultry industry to treat infections and promote growth has frequently been associated with increases in bacterial resistance to clinically important drugs [1, 2, 3, 4]. For instance, when fluoroquinolones are used, they may select for fluoroquinolone-resistant bacteria that can be found in poultry litter [5]. It is not known, however, whether all of the antimicrobial drugs that reach poultry litter persist indefinitely in that environment or are degraded by microorganisms that grow in the litter.

Norfloxacin, a fluoroquinolone antimicrobial agent, is used clinically for the treatment of urinary tract infections, bacterial enteritis, and eye infections [6, 7, 8]. The same drug is also used in poultry production in some countries for chronic respiratory diseases caused by *Mycoplasma synoviae* and *Escherichia coli* [9, 10, 11], although it is not registered for this purpose in the United States.

Poultry litter contains many bacteria, some of which may be resistant to multiple antibiot-

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ics [5, 12, 13]. Although *Enterococcus* spp., *Salmonella enterica*, and other pathogenic bacteria are sometimes found [13, 14, 15], they usually are minor components of the total poultry litter microbiota [16]. Saprobic fungi in the genera *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, and others are also found in poultry feed and litter [17, 18, 19, 20]. The objective of this study was to determine whether poultry litter materials could affect the degradation of norfloxacin by *Pestalotiopsis guepini*, a fungus known to metabolize fluoroquinolones [21].

MATERIALS AND METHODS

Typical poultry litter materials (10 g of rice hulls, 10 g of pine shavings, or 20 g of ground corncobs) were placed in 500-mL mason jars and sterilized by autoclaving for 1 h on each of 2 successive days. Cultures of the fungus *Pestalotiopsis guepini* P-8 (ATCC-MYA-1325), grown on petri dishes of potato dextrose agar, were macerated in sterile water using a blender. Each jar of litter material was inoculated with 10 mL of the blended mycelium. Norfloxacin was dissolved in 2% aqueous KOH (100 mg of norfloxacin per mL) and filter-sterilized, and then 1 mL of this solution was added to each jar together with 40 mL of sterile water. Controls were prepared using each of the types of litter material without the fungus or without norfloxacin. The jars were incubated in the dark at 28°C. On d 0, 10, 14, and 20, triplicate sets of all cultures and controls were harvested, filtered using glass wool, and extracted 3 times with 100 mL of methylene chloride. The extracts were combined and then evaporated in vacuo. The residues were dissolved in 2.0 mL of methanol for analysis.

The extracts were analyzed for possible norfloxacin metabolites by high-performance liquid chromatography (HPLC), using a Hewlett-Packard Series 1100 liquid chromatograph with a Prodigy 5 μm ODS(3) 10.0 × 250 mm column. The mobile phase consisted of a water and methanol gradient containing 0.2% acetic acid [22] at a flow rate of 3.5 mL/min. The diode array detector was monitored at 280 nm, and metabolite concentrations were estimated from the total peak areas.

Liquid chromatography and mass spectrometry analyses were performed on a Hewlett-Packard 5989B mass spectrometer equipped with an HP 1090L/M liquid chromatograph. A 2.0 × 250 mm Prodigy column was used with a water and acetonitrile gradient containing 0.1% formic acid [21]. Full scans were acquired in the positive-ion electrospray mode, with the capillary exit voltage at either +100 V or variable for molecular weight confirmation as the protonated molecule. The analysis was repeated at +200 V to obtain fragments with $[MH\text{-}H_2O]^+$ as the base peak. Norfloxacin metabolites were identified by comparing retention times and mass spectra with those published previously [21].

Proton nuclear magnetic resonance (^{1}H NMR) spectral analyses were performed at 500 MHz on a Bruker AM500 NMR spectrometer [22] using deuterated methanol as the solvent. The results were compared with those published previously [21].

RESULTS AND DISCUSSION

Pestalotiopsis guepini grew well on rice hulls and corncobs but not on pine shavings. At 20 d, 4 metabolite peaks were detected by HPLC in extracts from dosed rice-hull cultures that were not seen in extracts from control jars. Two of the metabolite peaks were also detected in extracts from dosed corncob cultures. At 10 and 14 d, smaller concentrations of the metabolites were observed. Some additional peaks were seen in the corncob and pine-shavings cultures and controls; the mass spectra of these peaks (not shown) indicated that they were not norfloxacin metabolites.

Mass and NMR spectroscopic analyses of the peaks collected as they eluted from the HPLC column were used to identify 4 norfloxacin metabolites in the cultures of *P. guepini* grown on rice hulls dosed with norfloxacin (Figure 1A); the mass and NMR spectra were the same as those published previously [21]. The structures of these compounds are shown in Figure 2. The major metabolite was 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (I), and the other metabolites were *N*-formylnorfloxacin (III), *N*-acetylnorfloxacin (IV), and desethylene-*N*-acetylnorfloxacin (II). Two norfloxacin metabolites were identified in cultures of *P. guepini* during growth on corncobs dosed with nor-

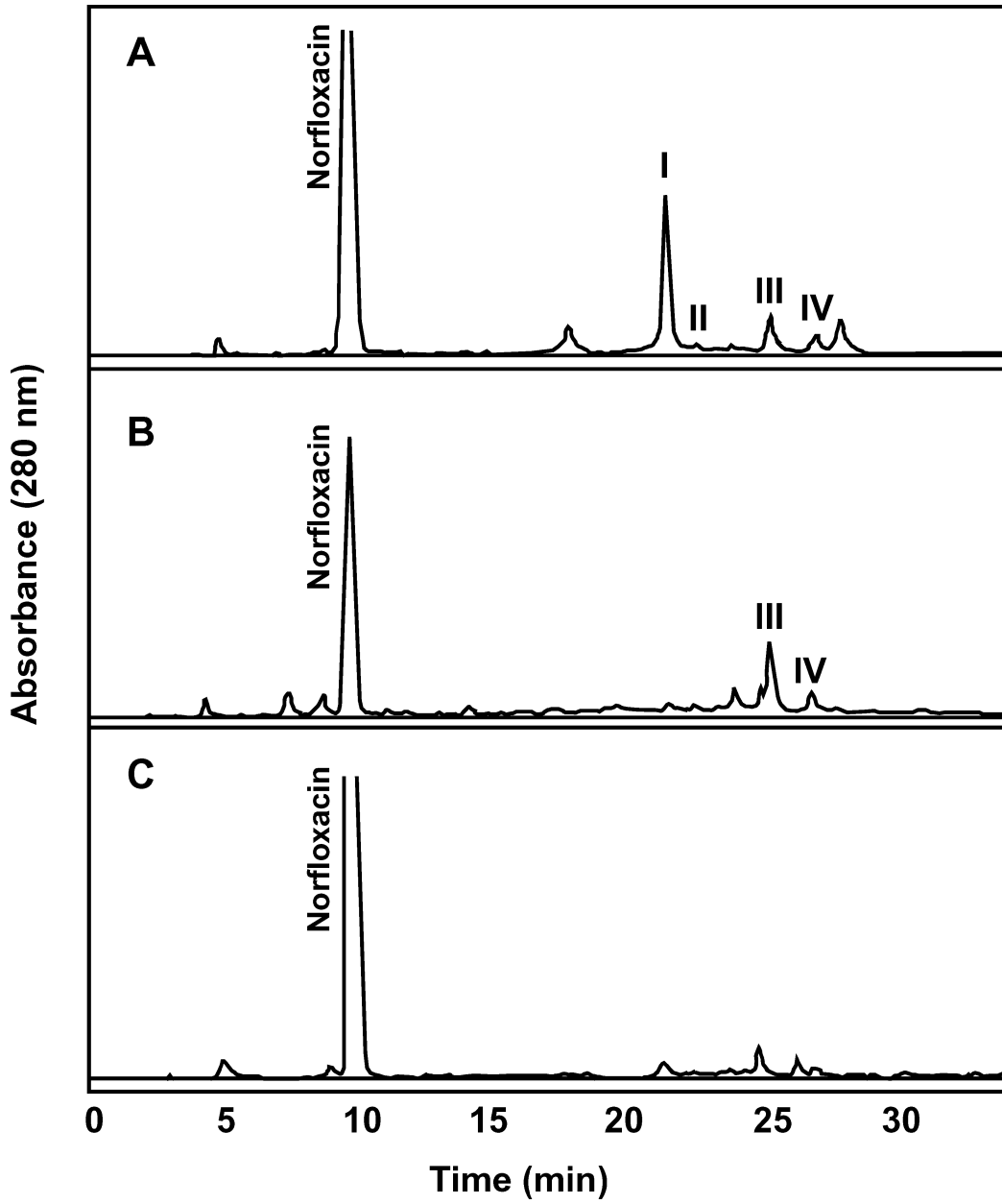

FIGURE 1. HPLC chromatograms (280 nm) of extracts from cultures of *Pestalotiopsis guepini* grown for 20 d on poultry litter materials dosed with norfloxacin, showing norfloxacin and fungal metabolites I to IV (A: rice hulls; B: corncobs; C: pine shavings). Peaks representing compounds that were shown by mass spectrometry to be unrelated to norfloxacin are not numbered.

floxacin (Figure 1B); the major metabolite was *N*-formylnorfloxacin (III), and the minor one was *N*-acetylnorfloxacin (IV). Little growth of the fungus was observed on pine shavings, and no norfloxacin metabolites were detected (Figure 1C).

The relative amounts of the norfloxacin metabolites produced on poultry litter materials at 20 d were estimated from the total areas of all the ultraviolet peaks in the HPLC chromatograms (Table 1). In rice-hull cultures, 18.4% of the total consisted of 7-amino-1-ethyl-6-

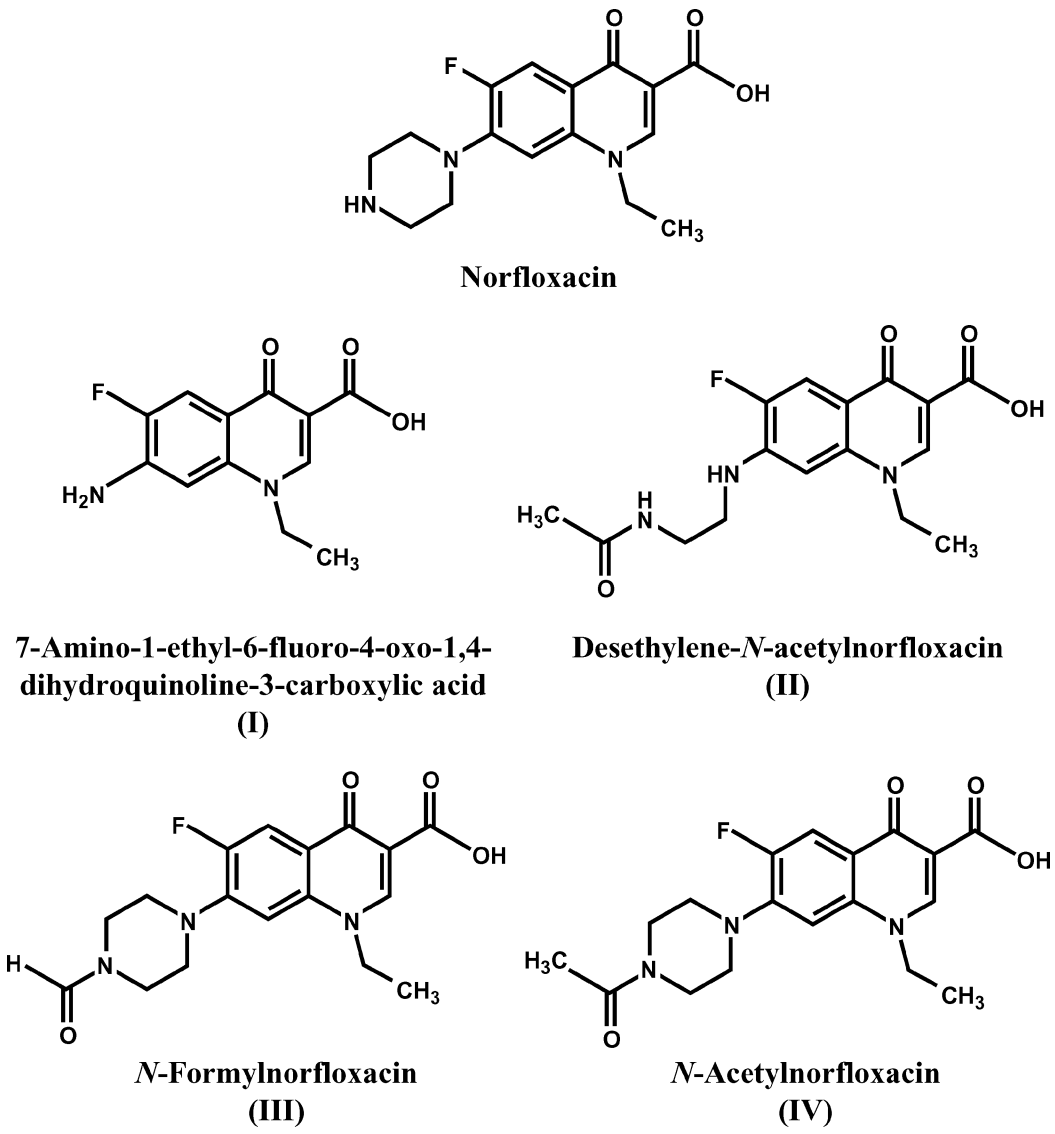

FIGURE 2. Structures of norfloxacin and the 4 metabolites produced from it by *Pestalotiopsis guepini* during growth on rice hulls dosed with norfloxacin [21].

fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (I), 0.5% was desethylene-*N*-acetylnorfloxacin (II), 4.2% was *N*-formylnorfloxacin (III), and 2.3% was *N*-acetylnorfloxacin (IV). In corncob cultures, 10.7% of the total was *N*-formylnorfloxacin (III), and 3.4% was *N*-acetylnorfloxacin (IV).

All 4 of the norfloxacin metabolites detected in poultry litter materials are also produced in sucrose-peptone broth cultures with norfloxacin [21], although *N*-acetylnorfloxacin is the predominant metabolite in broth cultures. In rice-hull cultures, more of metabolite I (7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), which has lost the piperazine ring, and less of metabolites II and IV were formed. In corncob cultures, metabolites III (*N*-formylnorfloxacin) and IV (*N*-acetylnorfloxacin) were found, but there were no products requiring cleavage of the piperazine ring. Little growth of *P. guepini* and no metabolites were observed on pine shavings,

TABLE 1. Amounts of each of the norfloxacin metabolites produced by *Pestalotiopsis guepinii* during growth for 20 d on poultry litter materials dosed with norfloxacin

	Percentage of total[A] represented by each metabolite			
Litter material	7-Amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (I)	Desethylene-*N*-acetyl-norfloxacin (II)	*N*-Formylnorfloxacin (III)	*N*-Acetylnorfloxacin (IV)
Rice hulls	18.4 ± 2.5	0.5 ± 0.0	4.2 ± 1.3	2.3 ± 0.8
Corncobs	ND[B]	ND	10.7 ± 3.8	3.4 ± 0.7
Pine shavings	ND	ND	ND	ND

[A]Means and standard errors from triplicate cultures, based on the total integrated areas at 280 nm of all of the identified metabolite peaks plus residual norfloxacin.
[B]ND = not detected.

which contain stilbenes, resin acids, and other compounds known to inhibit many fungi [23].

Another fungus, *Trichoderma viride*, metabolizes norfloxacin to the conjugate 4-hydroxy-3-oxo-4-vinylcyclopent-1-enylnorfloxacin when grown in sucrose-peptone broth [24] and to the same conjugate plus *N*-acetylnorfloxacin when grown on rice hulls [25]. The mammalian metabolites of norfloxacin include *N*-acetyl- and *N*-formylnorfloxacin, an ethylenediamine-substituted quinolone, an aminoquinolone, an oxonorfloxacin, a methyl ester, and an *N*-acetylethylenediamine-substituted quinolone [26]. Since the known metabolites of fluoroquinolones are generally less active as antibacterial agents than the parent drugs [27], the fungal transformation of fluoroquinolones in poultry litter materials may reduce the selective pressure on bacteria toward increased drug resistance. The type of litter materials used may affect the growth of fungi and the transformation of antimicrobial agents.

CONCLUSIONS AND APPLICATIONS

1. Two common poultry litter materials, rice hulls and corncobs, served as nutrients for the fungus *Pestalotiopsis guepini* and allowed it to transform added norfloxacin.
2. When grown on rice hulls dosed with norfloxacin, *P. guepini* produced 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, *N*-formylnorfloxacin, *N*-acetylnorfloxacin, and desethylene-*N*-acetylnorfloxacin. When grown on corncobs, the fungus produced only *N*-formylnorfloxacin and *N*-acetylnorfloxacin.
3. *Pestalotiopsis guepini* did not grow well on pine shavings, nor did it metabolize norfloxacin on them.

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