ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1989, p. 670–673
0066-4804/89/050670-04$02.00/0

Vol. 33, No. 5

Resistance to Antimicrobial Agents and Prevalence of R Plasmids in *Pasteurella multocida* from Turkeys

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One hundred fifty-three isolates of *Pasteurella multocida*, representing the causative agent of 95% of all known outbreaks of fowl cholera occurring in California meat and breeder turkeys from August 1985 through February 1987, were examined for susceptibility to antimicrobial agents. Of the 153 isolates, 6 were shown to be resistant to one or more antimicrobial agents. Of the six resistant isolates, five contained R plasmids. All but one of the R plasmids were small (6 to 7 megadaltons) and nonconjugative, encoding resistance to tetracycline or kanamycin, streptomycin, and sulfonamides; the other was large (70 megadaltons) and conjugative, transferring resistance to kanamycin, streptomycin, sulfonamides, and tetracycline to *P. multocida* and *Escherichia coli*. The three plasmids encoding resistance to tetracycline alone appeared identical.

Antimicrobial agents are used either to treat or to prevent infectious diseases. In the livestock industry, they are also used to improve both rate of weight gain and feed efficiency. The rationale for this latter use is vague, and its effectiveness is questionable. For whatever reason, the use of such agents places selective pressures on genes that encode resistance to antimicrobial agents.

We estimate that at least 60% of the 20 million turkeys raised in California for food each year receive antimicrobial agents (22; D. W. Hird, T. E. Carpenter, and K. P. Snipes, Proc. 37th Western Poultry Dis. Conf. 1988, p. 72). That this practice has selected resistant infectious agents affecting poultry has not been proven, but the isolation of multiply resistant strains of *Escherichia coli* and *Salmonella* spp. suggests that this has occurred (10, 11, 13).

Fowl cholera, produced by the gram-negative bacterium *Pasteurella multocida*, is a major infectious disease of turkeys. The disease occurs as either acute septicemia with high mortality or a more chronic condition affecting mainly the respiratory tract (20).

An integral part of the treatment of fowl cholera involves the use of antimicrobial agents. Resistance of *P. multocida* to commonly used drugs, e.g., sulfonamides and tetracycline, is thought to be uncommon, but differences between the methods used to measure susceptibility make conclusions difficult to draw, especially with respect to sulfonamides (9, 23). This pattern may be changing, however. A recent report from Australia indicated that 44% of *P. multocida* isolates from chickens were resistant to tetracycline (19).

Isolates of *P. multocida* resistant to antimicrobial agents have been shown to possess R plasmids that encode resistance to streptomycin, sulfonamides, and rarely to tetracycline (3, 15, 21). These plasmids have been shown to be small (less than 10 megadaltons [MDa]) and nonconjugative. A plasmid with a sex factor function has been reported in *P. multocida* and shown to be responsible for the transfer of a small, nonconjugative R plasmid to *E. coli* and other strains of *P. multocida* (14). Although the moles percent G+C contents of these R plasmids suggest that they are of enteric origin, they do not belong to a recognized compatibility group (15).

All previous studies of resistance to antimicrobial agents and the biology thereof have made use of *P. multocida* from isolated outbreaks occurring over many years from geographically diverse regions. There are no data available regarding the prevalence of resistant *P. multocida* strains responsible for fowl cholera together with information on the prevalence of R plasmids encoding such resistance. To gather such data, all isolates of *P. multocida* from tissues of a sample of California meat and breeder turkeys dead of fowl cholera from August 1985 to February 1987 were tested for susceptibility to antimicrobial agents. Those found to be resistant to one or more of the agents were studied further to determine whether they contained R plasmids responsible for the observed resistance.

MATERIALS AND METHODS

Bacterial strains. *P. multocida* isolates (n = 153) were obtained from tissues of turkeys dead of fowl cholera in California from August 1985 through February 1987. The turkeys from which the isolates had been obtained were submitted to the California State Veterinary Diagnostic Laboratory and represented 95% of all known outbreaks of fowl cholera during this period in California (based on processing records of the California Turkey Industry Federation). *E. coli* C600-1 (a nalidixic acid-resistant mutant of strain C600, *lac thr leu thi*; 1) and *P. multocida* P1059-1A (a nalidixic acid-resistant, plasmidless variant of strain P1059 belonging to somatic serotype 3) were used as recipients for conjugative transfer experiments.

Susceptibility testing. Isolates were tested for susceptibility to chloramphenicol, gentamicin, kanamycin, penicillin G, streptomycin, sulfonamides, tetracycline, and trimethoprim-sulfonamide (ratio of 1:20) by an agar dilution method (24). Mueller-Hinton agar was used for testing all of the antimicrobial agents, except for sulfonamides and trimethoprim-sulfonamide. The medium used for testing susceptibility to sulfonamides and trimethoprim-sulfonamide was Mueller-Hinton agar supplemented with laked horse blood (5%). The susceptibilities of transconjugants and transformants were determined by disk diffusion assay (2).

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TABLE 1. Susceptibility of *P. multocida* to antimicrobial agents[a]

Antimicrobial agent	Cumulative % of isolates susceptible to the following concn (μg/ml):									
	0.25	0.5	1	2	4	8	16	32	64	128
Chloramphenicol	79	99	99	99	99	100				
Gentamicin	0	0	28	53	100					
Kanamycin	0	0	0	1	49	75	81	81	81	100
Penicillin	99	99	99	100						
Streptomycin	0	0	0	0	9	70	96	97	97	100
Sulfonamides	0	0	0	11	37	67	82	96	98	98
Tetracycline	29	57	88	94	95	95	95	99	100	
Trimethoprim-sulfonamide[b]	100									

[a] Isolates were obtained from tissues of turkeys dead of fowl cholera (n = 153) in California in 1985 to 1987.
[b] This was tested at a 1:20 ratio; the results obtained with the trimethoprim fraction are shown.

Preparation of plasmid DNAs. A colony lysis technique was used to isolate plasmid DNAs for agarose gel electrophoresis (16). Purified plasmid DNAs were prepared by isopycnic centrifugation in a cesium chloride-ethidium bromide gradient (3, 12).

Transformation with plasmid DNAs. Purified plasmid DNAs were used to transform *E. coli* C600-1 (7). Transformants were selected on MacConkey agar containing the antimicrobial agent to which the isolate of *P. multocida* was resistant.

Conjugative transfer of plasmids. Conjugative transfer of plasmids was performed by using *E. coli* C600-1 and *P. multocida* P1059-1A as recipients (8). Selection of transconjugants was done on MacConkey agar (for *E. coli* C600-1) or brain heart infusion agar (for *P. multocida* P1059-1A) containing the antimicrobial agent to which the donor isolate of *P. multocida* was resistant and nalidixic acid.

Endonuclease digestion. The restriction endonuclease *Bst*UI (New England BioLabs, Inc., Beverly, Mass.) was used to digest cesium chloride-purified plasmid DNAs (17).

Agarose gel electrophoresis. The method used for agarose gel electrophoresis was that of Meyers et al. (18).

Serotyping. The somatic serotype was determined by the agar gel immunodiffusion method (5). The capsular type was determined by assessing the lability of the capsule to hyaluronidase (6). Possession of a capsule was determined by examining colonies growing on glucose starch agar under indirect light (4).

RESULTS AND DISCUSSION

During the time when the isolates were collected, 96 meat and breeder flocks were reported to be infected with *P. multocida*, placing approximately 3 million turkeys at risk. Since we monitored approximately 95% of all turkey flocks in California, these 96 infected flocks represented essentially all known outbreaks in California.

Of the 153 isolates of *P. multocida*, 6 (4%) were resistant to the drugs tested (Table 1). The drugs to which the six isolates were resistant included tetracycline, streptomycin, kanamycin, and sulfonamides (Table 2).

These 6 isolates contained plasmids (as did 28 of the nonresistant isolates), and 5 of the isolates contained R plasmids, as shown by transfer of resistance by plasmid DNA to *E. coli* by transformation (FC 30, FC 138, FC 144A, and FC 144B) or conjugation (FC 43). Attempts to demonstrate transfer of resistance from FC 30, FC 138, FC 144A, and FC 144B to *P. multocida* or *E. coli* by conjugation were unsuccessful. The 2.6-MDa plasmid in FC 11, did not transfer resistance to *E. coli* by transformation. Lack of a method to transform *P. multocida* precluded determination of transfer of resistance by this plasmid to *P. multocida*. Attempts to demonstrate transfer of resistance by this plasmid to *E. coli* or *P. multocida* by conjugation were unsuccessful. It should be noted that cryptic plasmids of this size are observed in approximately 22% of isolates from turkeys that have died of fowl cholera (unpublished data). The five R-plasmid-containing isolates belonged to somatic serotype 3,4 (FC 30, FC 138, FC 144A, and FC 144B) or 1,4 (FC 43) and were either of capsular type A (as were >98% of the 153 isolates studied [data not shown]) or nonencapsulated (FC 144A and FC 144B). All but one of the R plasmids were small and nonconjugative, encoding resistance to tetracycline (FC 138, FC 144A, and FC 144B; 6.2 MDa) (Fig. 1, lanes B to D and F to G) or streptomycin, sulfonamides, and kanamycin (FC 30; 6.5 MDa) (Fig. 1, lanes A and E). All of these tetracycline resistance plasmids appeared to be identical as determined by digestion with the restriction endonuclease *Bst*UI (Fig. 2). The R plasmid obtained from FC 43 was of higher molecular mass (70 MDa) and conjugative, capable of transferring resistance to tetracycline, streptomycin, sulfonamides, and kanamycin to *E. coli* and *P. multocida* at frequencies of 4.2×10^{-4} and 2.4×10^{-5} exconjugates per

TABLE 2. Antimicrobial agent susceptibility of resistant isolates of *P. multocida* isolates from turkeys

Isolate	MIC (μg/ml)							
	Chloramphenicol	Gentamicin	Kanamycin	Penicillin	Streptomycin	Tetracycline	Sulfonamides	Trimethoprim-sulfonamide[a]
FC 11	0.5	4	128	0.25	128	32	8	0.25
FC 30	0.25	4	128	0.25	128	1	256	0.25
FC 43	0.5	4	128	0.25	128	16	256	0.25
FC 138	0.25	2	8	0.25	8	32	8	0.25
FC 144A	0.25	2	8	0.25	8	32	16	0.25
FC 144B	0.25	2	8	0.25	8	32	4	0.25

[a] This was tested at a 1:20 ratio; the results obtained with the trimethoprim fraction are shown.

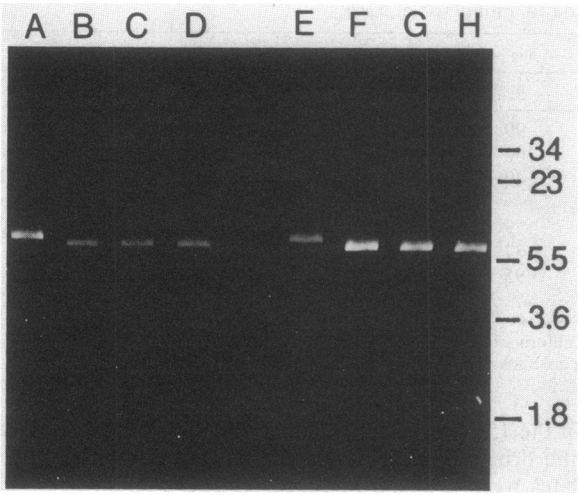

FIG. 1. Agarose gel electrophoresis of plasmid DNAs obtained from *P. multocida* isolated from turkeys with fowl cholera. Lanes: A, DNA from FC 30; B, DNA from FC 138; C, DNA from FC 144A; D, DNA from FC 144B. Lanes E to H contained plasmid DNAs obtained from *E. coli* C600-1 transformed with plasmids from FC 30, FC 138, FC 144A, and FC 144B, respectively. The numbers represent the positions of migration of plasmids of known molecular masses (megadaltons).

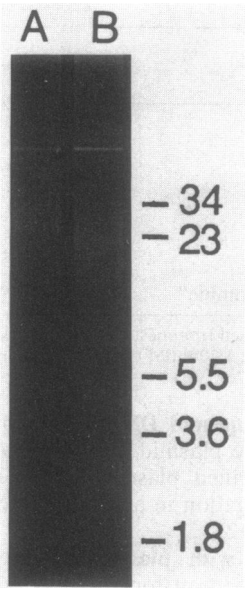

FIG. 3. Agarose gel electrophoresis of plasmid DNAs obtained from *P. multocida* FC 43 (lane A) and *P. multocida* P1059-1A following conjugation (lane B). The numbers represent the positions of migration of plasmids of known molecular masses (megadaltons).

donor, respectively (Fig. 3). The *P. multocida* P1059-1A exconjugate was serotyped to confirm that it was P1059-1A and not a nalidixic acid-resistant mutant of FC 43.

Isolates FC 138, FC 144A, and FC 144B are likely to be of the same strain, not only because they contained the same nonconjugal R plasmid but also because they were isolated from turkeys from the same flock on the same premises but at different times (7 November 1986 and 5 December 1986). Isolates FC 30 and FC 43 were obtained from turkeys on two other, unrelated farms.

Antimicrobial agents were used in two of the three flocks before the outbreak of fowl cholera. The flock from which FC 138, FC 144A, and FC 144B were obtained received a tetracycline and a sulfonamide. The flock from which FC 43 was obtained received a tetracycline, a sulfonamide, neomycin, and nitrofurazone. Isolate FC 30 was obtained from a flock with no history of use of antimicrobial agents before the outbreak. No conclusions can be drawn from these observations because of the small number of isolates.

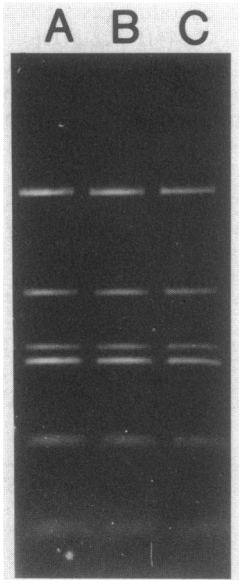

FIG. 2. Agarose gel electrophoresis of tetracycline resistance-encoding plasmids from FC 138, FC 144A, and FC 144B after digestion with *Bst*UI.

All but one of the R plasmids found in this study were small and nonconjugative. We are not aware that a conjugative R plasmid in *P. multocida* has been described previously. The fact that the conjugative R plasmid described herein was transferred not only to *E. coli* but to *P. multocida* as well is a finding of considerable significance. It has been difficult to explain the occurrence of the same R plasmid in different isolates of *P. multocida* unless it is hypothesized that the isolates were of the same strain (as the data suggest for FC 138, FC 144A, and FC 144B). A conjugative R plasmid, however, has the genetic information and the potential to transfer resistance to antimicrobial agents to susceptible *P. multocida* in vivo.

We conclude that resistant *P. multocida* from turkeys in California is uncommon. However, there is potential for spread of resistance, as evidenced by the finding of a strain of *P. multocida* containing a conjugative R plasmid.

ACKNOWLEDGMENTS

This work was supported by funds provided by the California Turkey Industry Federation and Livestock Disease Research Laboratory (University of California).

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