

# R Factor Transmission In Vivo<sup>1</sup>

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Experimental infections were induced in weanling pigs orally both with nalidixic acid (NA)-sensitive and -resistant strains of *Salmonella choleraesuis* var. *kunzendorf*, designated RC221 and RC221NA, respectively. Prior to the time of infection, cultures of normal bacterial flora were isolated from swine fecal matter and screened for the presence of R factors. A majority of these bacterial isolates harbored transferable resistances. Both strains RC221 and RC221NA have been shown to be competent recipients in vitro of the R factors present in the normal intestinal flora. The property of NA resistance greatly facilitated recovery of the infecting organism. After infection, salmonellae from liver, lung, spleen, lymph node, intestine, and feces were screened for the presence of R factors. Transfer of drug resistance in vivo was a rare occurrence; however, if infected specimens, particularly intestinal, were incubated in nutrient broth prior to plating, R factor transfer occurred, presumably in the test tube. Changes in recipient cultures were frequently observed after introduction of R factors from organisms of pig origin into the *S. choleraesuis* var. *kunzendorf* test organisms. Alterations include changes in typing reaction, granular growth in broth, differences in colony form, and reduction of virulence.

The occurrence of multiply drug-resistant bacteria among the normal enteric microflora of domestic animals has been reported (2, 6, 7). The drug resistance was infectious, since it could be transferred in mixed culture to other *Enterobacteriaceae*. The significance of such R factor-carrying bacteria as potential concern for the health of the public has remained unclear. It is known that transmission of R factors occurs in vivo (1, 8); however, it has usually been necessary to create conditions highly favorable to the formation of mating pairs to detect it. The frequency with which such transfers occur naturally, particularly to disease-causing bacteria, has been the subject of considerable speculation. The present report relates studies to assess the frequency of R factor transfer from the normal enteric microflora of swine to a virulent pathogen by using an experimental *Salmonella choleraesuis* infection of swine raised and maintained under farm conditions.

## MATERIALS AND METHODS

**Strains.** Nalidixic acid (NA)-sensitive and -resistant strains of *S. choleraesuis* var. *kunzendorf*, designated RC221 and RC221NA, respectively, were used to infect pigs orally via the feed. Both these strains were highly virulent for mice and for pigs; neither strain

harbored an R factor. Strain RC221 was isolated from a pig suffering from salmonellosis. Substrain RC221NA was derived from strain RC221 by selection on media containing 500 µg of NA per ml. Assays for the presence of transferable R factors were run by using as recipients a tryptophan-dependent substrain of *Escherichia coli* K-12 F<sup>-</sup> made resistant to 500 µg of NA per ml (K-12NA) as well as strains RC221 and RC221NA.

**Media.** Liquid cultures were prepared in Penassay Broth (Difco). All cultures were routinely tested for antibiotic sensitivity by the paper disc method on Penassay and Mueller Hinton Agar (Difco). All sensitivity discs were obtained from the Baltimore Biological Laboratories (BBL). MacConkey agar was used in all selective media except when sulfonamide (SU) resistance was being tested.

**Drugs.** The drugs used were sulfaethoxypyridazine (SE), chlortetracycline (A), sulfamethazine, nitrovin (American Cyanamid Co.); dihydrostreptomycin (DS; E. R. Squibb & Sons); and NA (Sterling-Winthrop Research Institute).

**Transfer of drug resistance in vitro.** The techniques used for qualitative and quantitative determinations of conjugal transfer of R factors in vitro were those of Watanabe (9).

**Drug-sensitivity test.** Approximately  $2 \times 10^4$  to  $5 \times 10^4$  colony-forming units (CFU) of an overnight culture of each strain to be tested were spread evenly over the surface of a dried agar plate, and discs containing drugs were then applied approximately equal distances apart. The plates were incubated at 37°C for 24 hr and read.

**Experimental infection.** Seven- to eight-week-old

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weanling pigs (average weight 25 lb) were infected orally with feed containing  $10^{10}$  to  $10^{11}$  CFU/animal.

**Bacteriological examination of clinical specimens.** Prior to infection and medication, organisms were isolated from swine fecal matter and assayed for the presence of transferable R factors. After infection, salmonellae from liver, lung, spleen, lymph node, intestine, and feces were screened for the presence of R factors by either directly swabbing infected specimens on selective media or first incubating them in broth prior to plating. At the termination of the experiment, cultures which were not *S. choleraesuis* var. *kunzendorf* were obtained from intestinal samples of surviving animals and tested for ability to transfer their resistances. When strain RC221 was used as the infecting organism, tetrathionate enrichment broth was used to enrich the salmonellae in infected specimens before plating on differential media. Since a selective force was not employed to recover RC221 organisms with transmissible resistances, isolates of RC221 were individually tested for R factors by pairwise mixed cultivations with *E. coli* K-12NA.

### RESULTS AND DISCUSSION

This report summarizes resistance transfer studies from three experiments in pigs. These were designed to determine the frequency of R factor-mediated drug-resistance transfer from normal gut microflora to a virulent pathogen in an infected animal under farm conditions.

The three experiments represented progressive refinements of the procedure. The first, involving 60 pigs, was conducted with *S. choleraesuis* var. *kunzendorf* RC221; in experiments 2 and 3 involving 40 and 60 pigs, respectively, an NA-resistant variant of *S. choleraesuis* var. *kunzendorf* (RC221NA) was utilized to facilitate recovery of the organism from infected animals. In experiments 2 and 3, the infected animals were treated with antibiotics (associated with R factor-mediated resistance) in the feed. None of the treatments used in experiment 2 was successful, but several treatments in experiment 3 were effective in preventing mortality. Therefore, in experiment 3, it was possible to study the influence of drug pressure on R factor transfer in vivo.

Strain RC221 does not harbor a transferable R factor (R<sup>-</sup>); however, it has been shown to be

a competent recipient of R factors in vitro and capable of transferring these acquired episomes to *E. coli* K-12NA. In the first experiment, 7 of 18 *E. coli* strains with R factors isolated from fecal matter of pigs prior to experimental infection with salmonella were shown to transfer their episomal resistances (A DS Su) into strain RC221. When RC221NA was employed as recipient, R factor transfer was demonstrated by 18 of 22 similar donor strains which harbored R factors; 16 of these 18 were *E. coli*.

The competency of strain RC221NA in vitro is shown in Table 1. *E. coli* donor strains P-10 and 105-106-1 (Table 1) were isolated from the intestines of pigs from experiments 1 and 2, respectively.

**Salmonella choleraesuis var. kunzendorf strain RC221 in pigs.** For experiment 1, an experimental infection was induced orally in weanling pigs with strain RC221. Prior to infection, 30 cultures were isolated from swine fecal matter. All isolates were assayed for the presence of transferable R factors by using as recipient *E. coli* K-12NA. Of the 30 isolates, 19 were *E. coli*; 18 of these harbored transferable drug resistance. Of these 18 isolates, 17 contained the same block of transferable resistance determinants (A DS Su).

A total of 217 *Salmonella choleraesuis* cultures were isolated from liver, lung, spleen, lymph node, intestine, and feces throughout the course of the experiment. One salmonella strain harbored an R factor (A DS) transferable to *E. coli* K-12NA; however, this was isolated from an uninfected, untreated animal and was serologically different from the infecting strain. All other isolates had antibiotic sensitivity patterns identical with the infecting strain.

A total of 38 cultures, which were not *S. choleraesuis*, were isolated from seven animals surviving at the termination of the experiment. Of the 38 cultures, 18 harbored transferable R factors (A DS); 11 of 38 were *E. coli* and, of these 11 cultures, 8 harbored the (A DS) R factor.

Although *S. choleraesuis* var. *kunzendorf* RC221 was a competent recipient of R factors in vitro, after an oral infection in pigs and isolation from

TABLE 1. *In vitro* frequency of conjugational transfer of R factors from *E. coli* of pig origin to RC221NA

Donor strain	R factor	Recipient	Selected by	Frequency of transfer of R factor <sup>a</sup>
105-106-1	DS Su	RC221NA	DS + NA	$3.0 \times 10^{-6}$
P-10	A DS Su	RC221NA	SE + NA	$2.4 \times 10^{-6}$
			A + NA	$6.0 \times 10^{-7}$

<sup>a</sup> The frequencies of transfer of R factors are expressed as the values per introduced donor cell after 30 min of mating.

a variety of tissues and from feces where competent donors of R factors were known to reside, no infecting organism was shown to have picked up a transferable R factor.

**Salmonella choleraesuis var. kuzendorf strain RC221NA in pigs.** Experiment 2 utilized NA-resistant *S. choleraesuis* RC221NA which greatly facilitated recovery of the infecting organism from specimens; direct platings of fecal material from uninfected pigs did not form bacterial colonies on NA medium. It was possible to screen large numbers of RC221NA cells for acquired resistance(s) by using agar medium supplemented with NA plus an antibiotic (A-type media) for which resistance was known to be R factor-mediated. The NA-resistant property of RC221NA was nontransferable. Reversion frequency from NA resistance to NA sensitivity was determined by the replica plating technique. None of 1,660 colonies examined had reverted to sensitivity. Strain RC221NA consistently formed equivalent numbers of colonies on medium with or without NA at 100  $\mu\text{g/ml}$ .

An experimental infection was induced orally in weanling pigs with strain RC221NA (experiment 2). Prior to infection and medication, two cultures were isolated from plates which had been inoculated with fecal matter of each of the 40 pigs. These 80 cultures were tested for susceptibility to a variety of chemotherapeutic agents by the paper disc method. One of these cultures (105-106-1) was studied in detail and shown to transfer part of its resistances (DS Su) to RC221NA in vitro (Table 1). Sixty-six of the cultures (approximately 83%) were shown to be resistant to one or more antibiotics, frequently associated with R factors. Specimens from large and small intestine, liver, lung, spleen, and lymph node were obtained at necropsy from the 30 infected pigs which either had died from infection or were killed at the termination of the experiment (28 days postinfection). Initially, infected specimens were screened for the presence of R factors by incubating them in Penassay Broth with or without NA prior to plating on A-type medium. The use of broth supplemented with NA at a level of 100  $\mu\text{g/ml}$  was unsatisfactory, because it allowed for the emergence of NA-resistant mutants among the normal bacterial flora of the intestines. Such mutations were not encountered when samples were plated directly on NA-containing agar. Transmission of R factors into RC221NA was demonstrated for samples taken from eight pigs which were incubated in broth prior to plating. Lactose-positive organisms with multiple drug resistance were isolated from these same broths. No R factor transmission was demonstrated when a number

of infected specimens were plated directly on A-type selective medium. The number of RC221NA organisms (without R factors) from some of these directly plated specimens was numerous as judged by confluent growth of this organism on NA medium without antibiotic.

Barbour (3) showed that NA inhibited episomal transfer if the donor was NA-sensitive. On this basis, direct platings on NA-containing medium would eliminate or greatly diminish any R factor transmission occurring on the plate, since the donor organisms present in the animals were NA-sensitive.

The third experimental infection was induced orally in weanling pigs with strain RC221NA to test the efficacy of a combination of A, sulfamethazine, and nitrovin. In this experiment involving 60 pigs, medication was initiated 3 days preinfection. The purposes of the resistance studies from this pig trial were threefold: (i) to determine the extent to which broth-incubated specimens give rise to R factor transmission in the test tube rather than in the animal, (ii) whether salmonellae with R factors could be recovered when specimens were plated directly on A-type selective medium, and (iii) to study the effect of drug pressure on in vivo R factor transfer.

Pigs were reared on the same farm as those used for experiments 1 and 2. Specimens from large and small intestine were obtained from 26 infected pigs from various treatment groups which had either died from infection or been killed at the termination of the experiment (28 days postinfection). Livers and spleens from 21 of these pigs were also sampled. Duplicate samples were obtained; one was incubated in broth prior to plating, and the other was plated directly on A-type selective medium (25  $\mu\text{g}$  of A plus 100  $\mu\text{g}$  of NA per ml and 200  $\mu\text{g}$  of DS plus 100  $\mu\text{g}$  of NA per ml). No resistant culture of the infecting organism was isolated from any directly plated specimen of liver or spleen. Specimens from the five remaining pigs were tested by direct plating to A-type selective medium only. These latter specimens did not produce colonies on any of the A-type selective media. RC221NA organisms which had acquired drug resistance were recovered from 28 of 74 specimens which were assayed by broth incubation for 24 hr prior to plating. These positive samples were obtained from 14 of the 21 animals tested. Platings of some specimens on A-type selective medium gave rise to numerous colonies; it was inferred that these colonies had received R factors in vitro. Antibiotic-resistant isolates from five of these specimens were subsequently shown to transfer their resistance(s) into *E. coli* strain

K-12NA. Lactose-positive organisms with identical multiple drug resistance patterns were also recovered from these broths.

Except for one specimen, obtained from the small intestine of a pig medicated with nitrovin, all direct platings on A-type selective medium were negative for salmonellae with R factors. Two colonies with resistance to DS Su were recovered from plates which had been directly swabbed with this specimen. One of these two isolates had altered properties characteristic of a rough culture. It produced rough colonies, formed granular growth on the bottom of the tube in liquid medium (leaving the broth transparent), no longer reacted serologically with antisera which had been specific for the organisms used to infect, and clumped in the presence of neutral acriflavine (4). This isolate was shown to transfer its resistances (DS Su) into *E. coli* K-12NA. Acquired resistances of the other isolate with smooth cultural characteristics were not transferred after 24 hr of mixed cultivation with *E. coli* K-12NA and subsequent plating on appropriate A-type selective medium. It has been shown that rough cultures of *S. choleraesuis* var. *kunzendorf* strain RC221NA exhibited both increased competence to receive R factors and reduced virulence for mice (5). Thus, of the two drug-resistant isolates recovered from one animal in the third pig experiment, one was rough and the other was unable to transfer its acquired resistances.

Survival data of experiment 3 are summarized in Table 2. Since effective drug therapy was achieved, it was of interest to study the influence of drug pressure on R factor transfer. All 20 animals medicated with a combination of A (100 g/ton) plus sulfamethazine (100 g/ton) survived the infection (Table 2). Some of these surviving animals were sacrificed, and samples of selected organs were plated out on NA and A-type medium. RC221NA organisms were not recovered from any of these samples.

In these three pig experiments, transfer of drug resistance *in vivo* was a rare event. It was frequently observed, after the introduction of different R factors into both RC221NA and RC221, that the organisms had altered cultural char-

TABLE 2. *Salmonella choleraesuis* var. *kunzendorf* RC221NA in pigs (pig experiment 3)

Treatment	Survivors/ total <sup>a</sup>
Noninfected controls	10/10
Infected-nontreated	3/10
Infected-medicated	
Nitrovin, 20 g/ton	5/10
Chlortetracycline, 200 g/ton	4/10
Chlortetracycline, 100 g/ton + Sulfamethazine, 100 g/ton	10/10
Chlortetracycline, 100 g/ton + Sulfamethazine, 100 g/ton + Nitrovin, 20 g/ton	10/10

<sup>a</sup> Twenty-eight days postinfection.

acteristics typical of a rough culture (5) and exhibited reduced virulence for mice. The significance of these alterations is currently under investigation.

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#### LITERATURE CITED

1. Akiba, T., K. Koyama, S. Kimura, and T. Fukushima. 1961. Studies on the mechanism of transfer of drug resistance in bacteria. VIII. Experiments on the transfer of drug resistance *in vivo*. *Med. Biol. (Tokyo)* 59:185-188.
2. Anderson, E. S., and N. Datta. 1965. Resistance to penicillins and its transfer in Enterobacteriaceae. *Lancet* 1:407-409.
3. Barbour, S. D. 1967. Effect of nalidixic acid on conjugational transfer and expression of episomal lac genes in *Escherichia coli* K12. *J. Mol. Biol.* 28:373-376.
4. Braun, W., and A. Bonestell. 1947. Independent variation of characteristics in *Brucella abortus* variants and their detection. *Amer. J. Vet. Res.* 8:386-390.
5. Jarolmen, H., and G. Kemp. 1969. Association of increased recipient ability for R factors and reduced virulence among variants of *Salmonella choleraesuis* var. *kunzendorf*. *J. Bacteriol.* 97:962-963.
6. Smith, H. W., and S. Halls. 1966. Observations on infective drug resistance in Britain. *Brit. Med. J.* 1:266-269.
7. Walton, J. R. 1966. Infectious drug resistance in *Escherichia coli* isolated from healthy farm animals. *Lancet* 2:1300-1302.
8. Walton, J. R. 1966. *In vivo* transfer of infectious drug resistance. *Nature* 211:312-313.
9. Watanabe, T. 1964. Selected methods of genetic study of episome-mediated drug resistance in bacteria. *Methods Med. Res.* 10:202-220.