

Transfer of Erythromycin Resistance from Poultry to Human Clinical Strains of *Staphylococcus aureus*

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The transfer of *ermA* and *ermC* genes, the two most common resistance determinants of erythromycin resistance, was studied with Luria-Bertani broth in the absence of additional Ca^{2+} or Mg^{2+} ions. Fifteen human and five poultry isolates of *Staphylococcus aureus*, which were resistant to erythromycin but carried different genetic markers for erythromycin resistance, were used for conjugation. Since both the donors ($\text{Amp}^{\text{S}}\text{-Tet}^{\text{r}}$) and recipients ($\text{Amp}^{\text{r}}\text{-Tet}^{\text{s}}$) were resistant to erythromycin, the transconjugants were initially picked up as ampicillin- and tetracycline-resistant colonies. The resistance transfer mechanisms of the chromosomally located erythromycin rRNA methylase gene *ermA* and the plasmid-borne *ermC* gene were monitored by a multiplex PCR and gene-specific internal probing assay. Four groups of transconjugants, based upon the transfer of the *ermA* and/or *ermC* gene, were distinguished from each other by the use of this method. Selective antibiotic screening revealed only one type of transconjugant that was resistant to ampicillin and tetracycline. A high frequency of transfer (4.5×10^{-3}) was observed in all of the 23 transconjugants obtained, and the direction of tetracycline and erythromycin resistance marker transfer was determined to be from poultry to clinical isolates. The transfers of the *ermA* and *ermC* genes were via transposition and transformation, respectively.

Staphylococcus aureus is an important cause of nosocomial as well as community-based infections. The use of antibiotics in humans, to treat infections, and in animals, to promote growth and prevent colonization by pathogenic bacteria, has led to an increased resistance among bacteria (2, 21, 25). The resistance often is transferable at interspecies and intergeneric levels (3, 18, 28). The relative ease with which bacteria become resistant to currently used antimicrobial agents is of concern to public health officials (8, 9, 31). The spread of resistance to antimicrobial agents in *S. aureus* is largely due to the acquisition of plasmids and/or transposons (19). Although transfer of resistance between staphylococcal strains in the laboratory has been shown to occur via transformation, transduction, and conjugation (6, 14, 15, 17, 35), only conjugative transfer appears to be significant in vivo (17, 35). In staphylococci, the conjugative transfer of resistance determinants is usually mediated by conjugative plasmids (5, 20, 38, 39) but has also been shown to occur in the absence of detectable conjugative plasmids (4). Conjugative plasmids, usually 35 to 50 kb (7), spread resistance determinants between species and genera (3, 17, 18, 28, 35). Besides transferring the resistance determinants, they can mobilize nonconjugative plasmids (5, 17), recombine with nonconjugative plasmids to form new plasmids (37), or acquire and transfer resistance transposons (36).

Studies with human staphylococcal strains indicate that *Staphylococcus epidermidis* is a reservoir of antibiotic resistance genes that can be transferred to *S. aureus* under in vitro and in vivo conditions (5, 10, 19, 20). Studies of drug resistance transfer between staphylococcal strains have been done mostly on human isolates; studies of transfer between animal and human staphylococcal strains are rare (16, 24), and little or no infor-

mation is available about the transfer of drug resistance between avian and human staphylococci. In the context of the prevalent use of antibiotics in the poultry industry and the limited data about the role of poultry staphylococcal isolates in drug resistance transfer, it is pertinent to ask whether poultry isolates also contribute to the spread of antibiotic resistance in human staphylococcal strains. The objective of this study was to investigate the role of poultry *S. aureus* isolates in the dissemination of antibiotic resistance to human clinical *S. aureus* isolates in vitro and the development of a method to study the resistance transfer between bacterial strains resistant to the same antibiotic. In this study, a PCR-based method is reported that was used in combination with the selective antibiotic screening method to study the direction and mechanism of resistance transfer between poultry and human staphylococcal isolates, both of which were resistant to erythromycin but carried different genetic markers.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Erythromycin-resistant *S. aureus* strains were isolated from the hock joints and internal organs of diseased chickens. These were identified using the Automicrobic System (BioMerieux Vitek, Inc., Hazelwood, Mo.) and maintained as in-house stocks (27). The clinical *S. aureus* strains were either from the University of Arkansas for Medical Sciences, Little Rock, or from the Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, D.C. All the isolates, which were highly resistant to erythromycin (MIC of $>256 \mu\text{g/ml}$), were stored in Luria-Bertani (LB) broth containing 20% glycerol at -70°C . Organisms were grown overnight at 37°C in LB broth or on tryptic soy agar plates supplemented with 5% sheep's blood (Remel, Lenexa, Kans.). The plasmids pEM9698 and pE194, both maintained in *Bacillus subtilis*, were used as controls for the detection of *ermA* and *ermC* genes, respectively.

Antibiotic resistance profile of bacterial strains. The antibiotic resistance profiles of various *S. aureus* strains obtained from poultry and human sources were determined by the disk-diffusion assay method (1). The diameter of the inhibition zone was measured in triplicate and interpreted according to standards set by the National Committee for Clinical Laboratory Standards (26). Ampicillin, penicillin, streptomycin, tetracycline, erythromycin, lincomycin, azithromycin, and ciprofloxacin were used for determining the sensitivity of the above strains.

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Transfer of antibiotic resistance in mixed liquid cultures. Bacterial cultures were grown for 12 h in LB broth at 30°C ($\sim 3 \times 10^9$ CFU/ml). Equal volumes (200 μ l) of the poultry strains (resistant to tetracycline) and clinical *S. aureus* cultures (resistant to ampicillin and ciprofloxacin but sensitive to tetracycline and streptomycin) were mixed in an Eppendorf tube. The cultures were supplemented with 200 μ l of LB broth and incubated at 37°C without shaking. Aliquots of 50 μ l each were withdrawn after 6 h and plated in triplicate on tetracycline (30 μ g/ml)- and ampicillin (100 μ g/ml)-tetracycline (30 μ g/ml)-containing hard agar plates after appropriate dilution. After 24 h, colonies resistant to ampicillin and tetracycline were picked up as transconjugants. The choice of these antibiotics was purposefully made so that the resistant colonies might represent the transconjugants where the transfer of ampicillin resistance from clinical to poultry strains or the transfer of tetracycline resistance from poultry to clinical strains had taken place. In either case, the transconjugants would be resistant to ampicillin and tetracycline. Although the transconjugants were initially picked up on these plates, the transfer mechanisms of the erythromycin resistance markers, *ermA* and *ermC* genes, were studied.

Isolation of DNA and gel electrophoresis. The total DNA from the overnight-grown cultures was isolated by the method of Thakker-Varia et al. (33), and the plasmid DNA was isolated by other methods (13, 30). The electrophoresis of plasmid DNA, total DNA (chromosomal and plasmid DNA), or their *Eco*RI digestion products was carried out on a 1.0% agarose gel. The gels were run at 40 mA for 4 h, stained with ethidium bromide, and photographed.

Detection of *ermA* and *ermC* genes by PCR and gene-specific probing. The *ermA* and *ermC* genes from the donors, recipients, and transconjugants were detected by multiplex PCR analysis using the gene-specific PCR primers (12). The transfer of these genes was studied by in-gel probing of the *Eco*RI-digested chromosomal DNA for the detection of *ermA* gene inserts or Southern blotting and hybridization of the total DNA for detection of the *ermC* gene by using gene-specific probes as described earlier (11, 12).

Frequency of transfer. The transfer frequency of tetracycline resistance was calculated as the ratio of ampicillin- and tetracycline-resistant cells (average of three platings) to the total number of tetracycline-resistant cells (average of three platings).

RESULTS

Antibiotic resistance profiles of poultry, clinical, and transconjugant strains. The poultry isolates were sensitive to ampicillin, penicillin, and ciprofloxacin but resistant to streptomycin, tetracycline, erythromycin, lincomycin, and azithromycin (Fig. 1A). The clinical strains were sensitive to streptomycin and tetracycline but resistant to the other six antibiotics (Fig. 1B). After mixed culture transfer experiments and plating on ampicillin (100 μ g/ml)-tetracycline (30 μ g/ml)-containing plates, 23 transconjugants were obtained. The resistance profiles of the transconjugants (Fig. 1C) were similar to those of the clinical strains (Fig. 1B), except that the transconjugants were resistant to tetracycline.

DNA profiles of the donors, recipients, and transconjugants. All of the poultry strains contained plasmids ranging from 1.6 to 8.0 kb in size (Fig. 2A). The clinical strains, on the other hand, contained no plasmids (Fig. 2B). DNA analysis of the transconjugants revealed that only 8 of the 23 transconjugants contained plasmids (Fig. 2C) and the other 15 did not (data shown for only two transconjugants). This observation suggested that there were at least two types of transconjugants, ones that had acquired the plasmid DNA and others that had not.

Multiplex PCR and the direction of resistance transfer. The antibiotic resistance and the DNA profiles of the transconjugants were helpful in screening and differentiating the transconjugants, but they did not reveal any information about the transfer of either *ermA* or the *ermC* gene. To determine whether the transfer of the chromosomally located *ermA* and the plasmid-borne *ermC* gene had taken place or not, the bacterial lysates from parents and transconjugants were subjected to multiplex PCR analysis to determine the presence of the two genes. The PCR revealed that the poultry strains had both the *ermA* and the *ermC* genes (Fig. 3A, lanes 5 to 9). The clinical strains, on the other hand, had only the *ermA* gene, except for strains 712, 716, and 803, which had neither *ermA* nor the *ermC* gene (Fig. 3A, lanes 18, 20, and 25). The

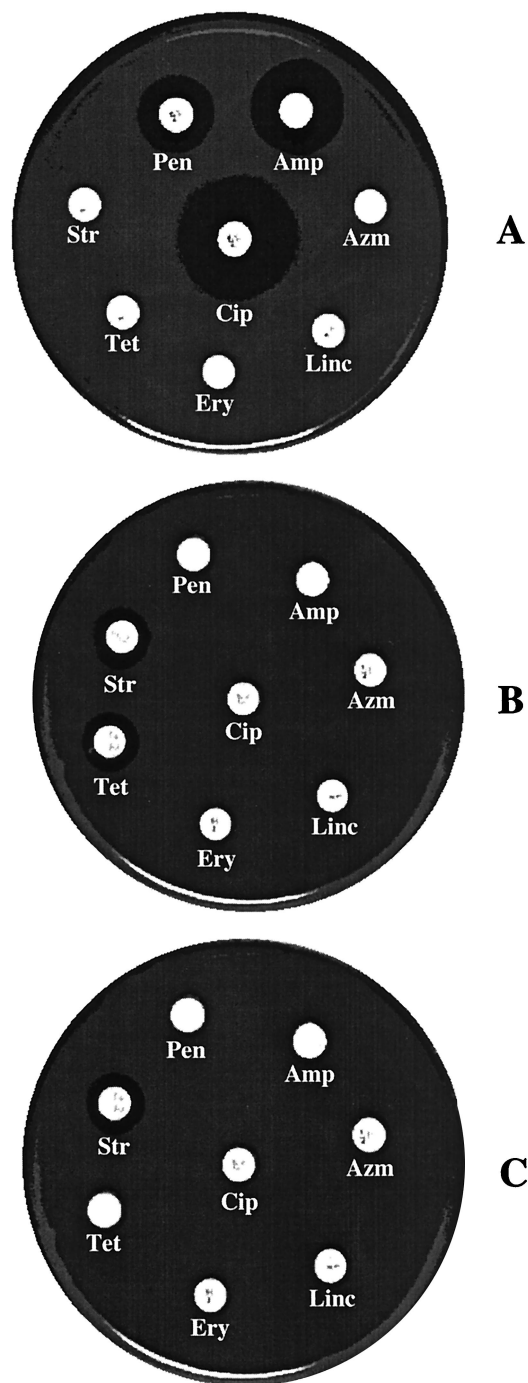


FIG. 1. Antibiotic resistance and sensitivity profile of the donors, recipients, and transconjugants. The strains were tested against eight different antibiotics. The antibiotic resistance profiles of the representative strains are shown for poultry isolates (A), clinical isolates (B), and transconjugants (C). The antibiotics and concentrations were as follows: ampicillin (Amp; 10 μ g/ml), penicillin (Pen; 10 U/ml), streptomycin (Str; 10 μ g/ml), tetracycline (Tet; 30 μ g/ml), erythromycin (Ery; 15 μ g/ml), lincomycin (Linc; 2 μ g/ml), azithromycin (Azm; 15 μ g/ml), and ciprofloxacin (Cip; 5 μ g/ml).

transconjugants that had acquired the plasmid DNA had also acquired the *ermC* gene (Fig. 3B, lanes 18 to 25). The transconjugants that did not acquire the plasmid DNA were also missing the *ermC* gene (Fig. 3B, lanes 2 to 16). All the transcon-

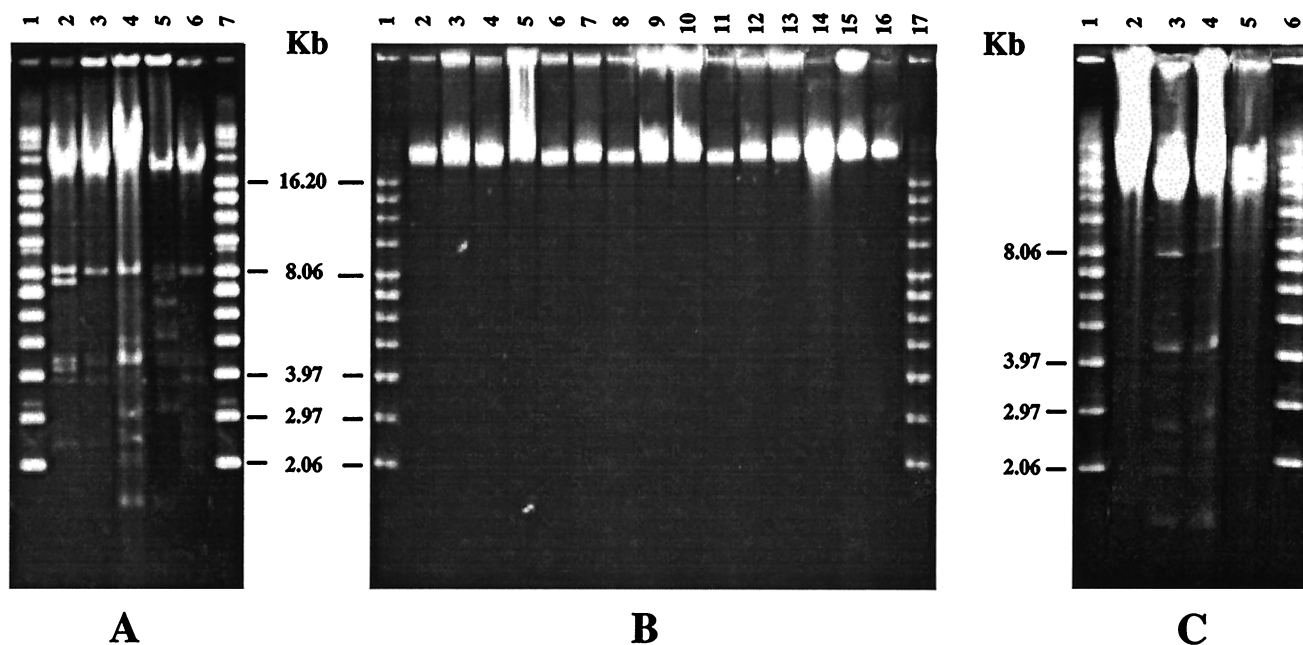


FIG. 2. Total DNA profile of the poultry and clinical *S. aureus* isolates. Approximately 1 μ g (5 μ l) of total DNA was loaded on 1.0% agarose gels and electrophoresed. The gels were stained with ethidium bromide and photographed. The letters p and c followed by numbers represent poultry and clinical strains, respectively. (A) DNA from poultry isolates. Lanes 1 and 7, supercoiled DNA ladder; lane 2, p45; lane 3, p46; lane 4, p58; lane 5, p62; lane 6, p63. (B) DNA from clinical isolates. Lanes 1 and 17, supercoiled DNA ladder; lane 2, c16; lane 3, c18; lane 4, c29; lane 5, c656; lane 6, c657; lane 7, c660; lane 8, c661; lane 9, c712; lane 10, c714; lane 11, c716; lane 12, c720; lane 13, c722; lane 14, c772; lane 15, c796; lane 16, c803. (C) DNA from transconjugants. Lanes 1 and 6, supercoiled DNA ladder; lane 2, c716; lane 3, p58; lane 4, p58-c716; lane 5, p58-c803.

jugants, however, possessed the *ermA* gene (Fig. 3B, lanes 2 to 16). The above observations suggested that the transfer of these markers occurred from poultry to clinical isolates.

Based upon the presence or absence of the *ermA* and/or *ermC* gene, the transconjugants were classified into four groups (Table 1). The transconjugants that belonged to group 1 possessed the *ermA* gene (Fig. 3B, lanes 2 to 12). The group 2 transconjugants also had the *ermA* gene (Fig. 3B, lanes 13 to 16), but the DNA-recipient parent strains did not have the *ermA* gene (Fig. 3A, lanes 18, 20, and 25). The third group of transconjugants showed the presence of both the *ermA* and the *ermC* genes (Fig. 3B, lanes 18 to 24). The only transconjugant strain that belonged to group 4 also had the *ermA* and the *ermC* genes (Fig. 3B, lane 25), but like group 2 transconjugants, the recipient parent did not have either of the two genes (Fig. 3A, lane 20).

In-gel and Southern hybridization. To determine the locations of the *ermA* and/or *ermC* gene, each group of transconjugants was probed with gene-specific internal probes for *ermA* and *ermC* genes. To determine the copy number of the *ermA* gene, *EcoRI*-digested chromosomal DNA molecules from the donors, recipients, and transconjugants were separated on agarose gels and subjected to in-gel probing with an *ermA* gene-specific internal oligonucleotide probe. The avian isolates (donors) had only two inserts, of 8.0 and 6.2 kb each (data shown for p58 only), which hybridized with the *ermA* gene-specific probe (Fig. 4A, lane 2), whereas the clinical isolates (recipients) had three to four copies (Fig. 4A, lanes 3 and 7). The recipients that did not test positive for the *ermA* gene by PCR also did not show any hybridization signal with the *ermA* gene-specific probe (Fig. 4A, lanes 5 and 9). All the transconjugants, however, had acquired an extra copy of the *ermA* gene (Fig. 4A, lanes 4, 6, 8, and 10) as 3.1-kb chromosomal DNA inserts

(data shown for only one representative from each group of transconjugants).

To determine which plasmid harbored the *ermC* gene, the DNA gel was blotted onto a nylon membrane and probed with an *ermC* gene-specific internal oligonucleotide probe. The probe hybridized with a 2.5-kb plasmid in group 3 and 4 transconjugants (Fig. 4B, lanes 19 to 24), suggesting that the *ermC* gene was present on this plasmid only. The transconjugants belonging to groups 1 (Fig. 4B, lanes 7 to 15) and 2 (Fig. 4B, lanes 16 to 18) showed no hybridization signal with the *ermC* gene-specific internal probe. No hybridization signal was detected with the chromosomal DNA in any group of transconjugants (Fig. 4B, lanes 2 to 24).

Frequency and efficiency of transfer of genetic markers. Based upon the antibiotic resistance phenotype and the PCR data, the transfer of tetracycline resistance and the two major determinants of erythromycin resistance, namely, *ermA* and *ermC*, occurred from poultry to clinical isolates. The efficiency of transfer was, however, different for different genetic markers. The transfer frequency of tetracycline resistance was calculated to be 4.5×10^{-3} . Since all the 23 transconjugants that were resistant to tetracycline also acquired an extra copy of the *ermA* gene, it was assumed that the transfer frequency of the *ermA* gene was at least equal to that of tetracycline resistance. The *ermC* gene was present in only 8 of the 23 transconjugants, and therefore, its transfer frequency appears to be three times lower than that for *ermA* or *tet*.

DISCUSSION

The ampicillin and tetracycline resistance phenotype of the transconjugants could arise by the transfer either of ampicillin resistance from clinical to poultry strains or of tetracycline

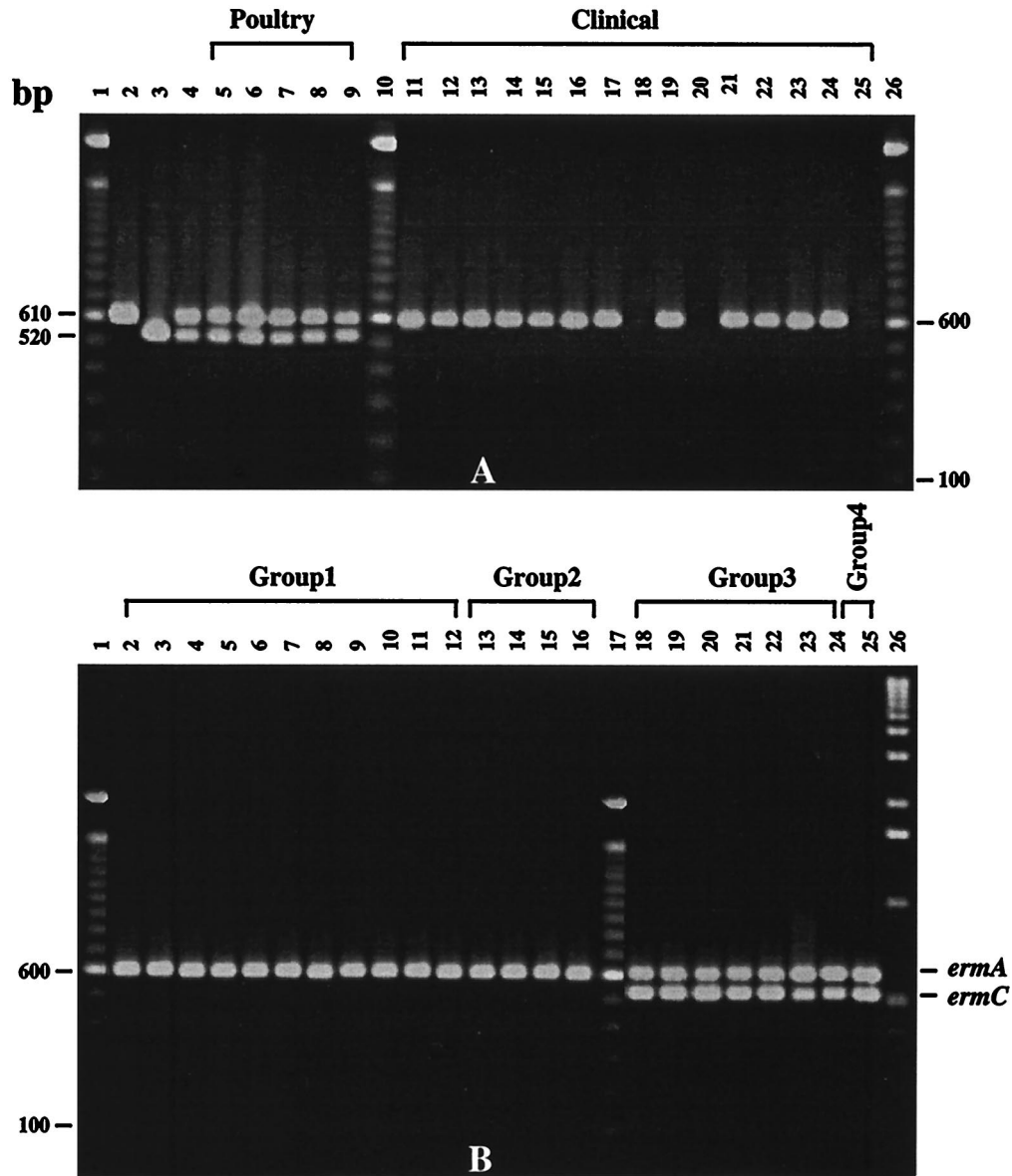


FIG. 3. Multiplex PCR analysis of the donors, recipients, and transconjugants. PCR samples (5 μ l) from different bacterial strains were loaded on a 1.0% agarose gel, and the gels were photographed after staining with ethidium bromide. (A) Lanes 1, 10, and 26, 100-bp DNA ladder; lane 2, *ermA* control from plasmid pEM9698; lane 3, *ermC* control from plasmid pE194; lane 4, *ermA-ermC* control; lane 5, p45; lane 6, p46; lane 7, p58; lane 8, p62; lane 9, p63; lane 11, c16; lane 12, c18; lane 13, c29; lane 14, c656; lane 15, c657; lane 16, c660; lane 17, c661; lane 18, c712; lane 19, c714; lane 20, c716; lane 21, c720; lane 22, c722; lane 23, c772; lane 24, c796; lane 25, c803. (B) Lanes 1, 17, and 26, 100-bp DNA ladder; lane 2, p45-c16; lane 3, p45-c18; lane 4, p45-c772; lane 5, p58-c657; lane 6, p62-c656; lane 7, p62-c657; lane 8, p62-c660; lane 9, p62-c661; lane 10, p62-c720; lane 11, p62-c722; lane 12, p63-c18; lane 13, p46-c716; lane 14, p58-c803; lane 15, p62-c712; lane 16, p63-c716; lane 18, p45-c29; lane 19, p45-c722; lane 20, p46-c714; lane 21, p46-c796; lane 22, p58-c18; lane 23, p58-c714; lane 24, p63-c29; lane 25, p58-c716. The transconjugants are identified as donor-recipient pairs. The transconjugants belonging to different groups are indicated. Designations beginning with "p" are for poultry strains; those beginning with "c" are for clinical strains.

TABLE 1. Transfer frequency of tetracycline resistance and the presence of *ermA* and *ermC* genes in transconjugants

Transconjugant	Transfer frequency ^a (10 ⁻³)	<i>ermA</i>	<i>ermC</i>
Group 1 ^b			
p45-c16	4.12	+	-
p45-c18	4.56	+	-
p45-c772	4.32	+	-
p58-c657	4.51	+	-
p62-c656	4.78	+	-
p62-c657	4.66	+	-
p62-c660	4.58	+	-
p62-c661	4.90	+	-
p62-c720	4.05	+	-
p62-c722	4.02	+	-
p63-c18	4.00	+	-
Group 2 ^c			
p46-c716	4.22	+	-
p58-c803	4.72	+	-
p62-c712	4.85	+	-
p63-c716	4.11	+	-
Group 3 ^d			
p45-c29	4.32	+	+
p45-c722	4.50	+	+
p46-c714	4.35	+	+
p46-c796	4.12	+	+
p58-c18	4.22	+	+
p58-c714	4.81	+	+
p63-c29	4.10	+	+
Group 4 ^e			
p58-c716	4.22	+	+

^a The transfer frequency represents the average of three plating experiments.

^b The recipients had multiple inserts of the *ermA* gene but acquired an extra copy of the gene.

^c The recipients had neither the *ermA* nor the *ermC* gene but acquired the *ermA* gene.

^d The recipients had *ermA* but acquired the *ermC* gene and an extra copy of the *ermA* gene.

^e The recipient acquired both *ermA* and *ermC* genes.

resistance from poultry to clinical isolates. Upon comparison, the antibiotic resistance and sensitivity profile of the transconjugants was found to be similar to that of the clinical strains, except that the transconjugants were resistant to tetracycline. The sensitivity of transconjugants to streptomycin and their resistance to ciprofloxacin suggested that they had more in common with the clinical isolates than with the poultry isolates. The clinical strains appeared to have acquired the tetracycline resistance from the poultry isolates. In the event of transfer from clinical to poultry isolates, the ampicillin, penicillin, and ciprofloxacin resistance has to have been transferred in order to explain the resistance and sensitivity profile of the transconjugants. Further, the sensitivity of the transconjugants to streptomycin could not be explained if the transfer of resistance(s) occurred from clinical to poultry strains. The direction of tetracycline resistance transfer was, therefore, determined to be from poultry to clinical *S. aureus* isolates.

The use of ampicillin and tetracycline as selective antibiotics was helpful in determining the direction of tetracycline resistance transfer but it was of no use in determining if the transfer of erythromycin resistance markers, *ermA* and *ermC*, between poultry and clinical strains had also occurred. Both the poultry and the clinical strains were resistant to high concentrations of erythromycin (MIC of >256 µg/ml). Based upon the total

DNA analysis of the transconjugants, only two types of transconjugants, the ones that acquired the plasmids and the ones that did not, were obtained. The PCR amplification of the *ermA* and *ermC* genes, however, indicated four types of transconjugants. The hybridization of a 3.1-kb insert with the *ermA* gene-specific probe in all the transconjugants and the presence of the *ermC* gene bearing plasmids in group 3 and 4 transconjugants also confirmed the PCR data. The use of a selective antibiotic in determining the resistance transfer between the donors and the recipients that show resistance to the same antibiotic is of little or no use. The use of PCR and gene-specific probing along with the selective antibiotic screening for the transconjugants is not only useful in determining the direction of resistance transfer but also helpful in determining the mechanism of resistance transfer.

The transfer of drug resistance in mixed liquid cultures was initially thought to require phage mediation and Ca²⁺ or Mg²⁺ ions (34), but it was shown later on that it can also occur in the absence of the phage (6, 22). The absence of externally added metal ions and transducing phages in our experiments, however, excluded the possibility of phage-mediated conjugation and transduction and pointed toward a phage-independent transfer mechanism(s). The transfer of the chromosomally located *ermA* gene, which is known to be associated with transposon Tn554 (29), suggested the possibility of transposon-mediated drug resistance transfer. The presence of an extra 3.1-kb chromosomal DNA insert in all the transconjugants confirmed that the transposition of the *ermA* gene to a different site on the recipient's chromosome had taken place. Our results are similar to those of an earlier study (32) in which the transposition of chromosomal gene markers was observed, but unlike our observations, the transfer was achieved during filter mating only and not in mixed culture transfer experiments.

Although the transfer of the *ermA* gene was shown to occur via transposition, the transfer of the plasmid-based *ermC* gene could not be explained by this mechanism. If transposition was responsible for the mobilization of smaller plasmids, all the transconjugants would have acquired the *ermC* gene. Since the transposition of the *ermA* gene took place in all the transconjugants and only 8 of the 23 transconjugants acquired the *ermC* gene, transposition does not seem to be responsible for the transfer of *ermC*. The involvement of conjugative plasmids that are known to mobilize the smaller plasmids was also ruled out because of our repeated failure to isolate larger conjugative plasmids by known techniques (13, 30). In the absence of detectable conjugative plasmids and phage-mediated conjugation, transformation seems to be the most likely mechanism for the transfer of the *ermC* gene. In fact, in an earlier mixed liquid culture transfer study (23), the transfer of smaller plasmids bearing penicillin resistance was observed in the absence of Ca²⁺ and transformation was believed to be the mechanism of transfer. Since our results were also similar to those of the above study, the transfer of smaller plasmids is believed to take place via transformation.

The demonstration that the poultry *S. aureus* strains can transfer the resistance to human *S. aureus* strains indicates that the strains belonging to different ecosystems can contribute to the spread of antibiotic resistance genes. Up until now, the transfer of resistance was studied between donors and recipients that were resistant to different antibiotics. The data presented in this study clearly demonstrate the usefulness of PCR and gene-specific probing along with the conventional selective antibiotic screening method to study the transfer of drug resistance between organisms that are resistant to the same antibiotic but carry different genetic markers. The method presented here offers a unique approach to analyze the

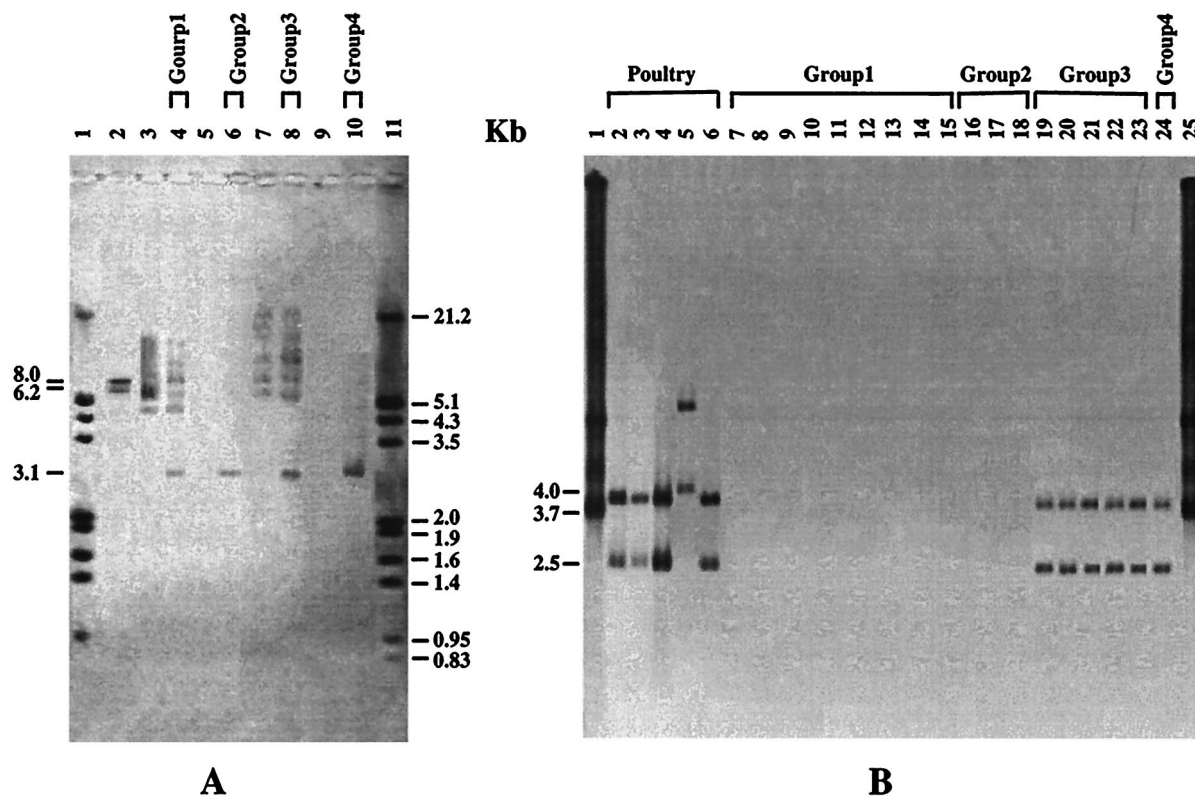


FIG. 4. In-gel and Southern hybridization. (A) *Eco*RI-digested DNAs of the donors, recipients, and transconjugants were probed with an *ermA* gene-specific internal oligonucleotide probe. Lanes 1 and 11, digoxigenin-labeled DNA molecular size marker III (Boehringer Mannheim); lane 2, p58; lane 3, c657; lane 4, p58-c657; lane 5, c803; lane 6, p58-c803; lane 7, c18; lane 8, p58-c18; lane 9, c716; lane 10, p58-c716. (B) Southern hybridization of the donor and transconjugant total DNA with an *ermC* gene-specific internal oligonucleotide probe. Lanes 1 and 25, control plasmid pE194 DNA; lane 2, p45; lane 3, p46; lane 4, p58; lane 5, p62; lane 6, p63; lane 7, p45-c16; lane 8, p45-c772; lane 9, p58-c657; lane 10, p62-c656; lane 11, p62-c660; lane 12, p62-c661; lane 13, p62-c720; lane 14, p62-c722; lane 15, p63-c18; lane 16, p46-c716; lane 17, p58-c803; lane 18, p62-c712; lane 19, p45-c29; lane 20, p45-c722; lane 21, p46-c796; lane 22, p58-c714; lane 23, p63-c29; lane 24, p58-c716. Designations are explained in the legend to Fig. 3.

transconjugants and could be extended to other such systems. By the use of this procedure, the direction of resistance transfer was clearly established to be from avian to human isolates of *S. aureus*. The transfer of resistance was found to be via transposition and transformation.

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