

Molecular Analysis of Human, Porcine, and Poultry *Enterococcus faecium* Isolates and Their *erm(B)* Genes

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Fifty-nine *erm(B)*-positive *Enterococcus faecium* strains isolated from pigs, broilers, and humans were typed using multilocus sequence typing (MLST), and the coding sequence of the *erm(B)* gene was determined. Identical *erm(B)* gene sequences were detected in genetically unrelated isolates. Furthermore, genetically indistinguishable strains were found to contain different *erm(B)* alleles. This may suggest that horizontal exchange of the *erm(B)* gene between animal and human *E. faecium* strains or the existence of a common reservoir of *erm(B)* genes might be more important than direct transmission of resistant strains.

Macrolide, lincosamides, and streptogramin (MLS) antibiotics may be important as alternative therapy for treatment of enterococcal infections in humans (19, 21). Acquired resistance to these antibiotics has frequently been described in enterococci originating from humans as well as from animals (4, 17, 21). The *erm(B)* gene conferring cross-resistance against MLS antibiotics is very common among human, porcine, and poultry enterococci (1, 12, 17, 21, 22).

Two different routes of antimicrobial drug resistance transfer from animal to humans are of importance, a direct and an indirect way of transfer. Direct transmission of resistance occurs when resistant zoonotic bacteria infect humans. In the indirect way, resistant bacteria originating from animals transfer their resistance genes horizontally to the human bacterial population. This occurs when animal-derived strains are able to survive, at least temporarily, in human sites, primarily the gut (1, 7, 9, 15).

This study was undertaken to obtain better insights into transmission dynamics of macrolide resistance genes between human and animal *Enterococcus faecium* strains, focusing on the hospital environment and pigs and broilers, respectively. The possible linkage to other genes was likewise investigated. For this purpose, macrolide- and lincosamides-resistant, *erm(B)*-positive *E. faecium* strains isolated from pigs, broilers, and humans were typed using multilocus sequence typing (MLST), and the coding sequence of their *erm(B)* gene was determined. Furthermore, the presence of the *vanA* and *aphA-3* genes, encoding vancomycin and aminoglycoside resistance, respectively, was determined to study genetic linkage of multiple resistance genes in macrolide resistant *E. faecium* strains.

A total of 59 *erm(B)*-positive *E. faecium* strains isolated from humans (19 strains), pigs (19 strains), pork carcasses (4

strains), and broilers (17 strains) were used in this study (Table 1). Twelve human isolates (H) were obtained from different patients in one hospital. The other human strains were isolated from seven different nonhospitalized (NH) (hemodialysis) patients and were collected in the same hospital. The porcine isolates all originated from different farms and slaughterhouses. The poultry isolates originated from nine different farms (Farm A to I). The isolates were identified as *E. faecium* using a PCR described by Kariyama et al. (14). The detection of the *erm(B)* gene was performed with a PCR described by Martel et al. (16).

MLST was performed as described by Homan et al. (10). The eBURST program was used to assess the genetic relationships of genotypes, to assign isolates to genetic complexes, and to study patterns of evolutionary descent of isolates within a complex (8). Sequence types were also compared with a database of MLST data from 139 epidemiologically unlinked *E. faecium* isolates from humans and livestock in The Netherlands, Australia, United Kingdom, United States, and France (<http://efaecium.mlst.net>) (10).

The coding sequence of the *erm(B)* gene was amplified using primers ORR1, 5'-ATGAACAAAAATATAAAATATT-3', and ORR2, 5'-TTATTTCTCCCGTTAAA-3'. The amplicon was sequenced using primers E1, 5'GAAAAGA/GTACTCAACCAAATA3'; E2, 5'AGTAACGGTACTTAAATTGTTTAC3'; E3, 5'CCATACCACAGATGTTCCAG3'; and E4, 5'AGATAGATGTCAGACGCACG3' (18).

The primers and the PCR running conditions for the detection of the resistance genes *aphA-3* and *vanA* were as described elsewhere (5, 20). To determine whether the *erm(B)* gene was linked with the *vat(E)* gene, a PCR was used with primers described by Jensen et al. (13).

Application of MLST revealed 42 different sequence types (STs) among the 59 isolates. eBURST analysis clustered the STs into three major complexes, three minor complexes, and 14 singletons (Fig. 1) (Table 1). Clonal complex (CC) 9 with presumed ancestral type ST-9 consists of 10 STs, representing 13 isolates from broilers and 2 isolates from humans (one H and one NH) and could be considered a poultry-specific CC. In

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TABLE 1. *erm*(B) allele and MLST data of the 59 *E. faecium* isolated recovered from humans, pigs, and broilers^a

<i>erm</i> (B) allele	Strain	Host	Source	Farm	MLST allelic profile						ST	CC	Genogroup		
					<i>adk</i>	<i>atpA</i>	<i>ddl</i>	<i>gyd</i>	<i>gdh</i>	<i>purK</i>				<i>pstS</i>	
<i>erm</i> (B-1)	HE28	Human (NH)	Stool		5	5	3	1	1	9	1	37	5	A	
	AME31	Pig	Colon		5	3	5	1	1	3	1	139	D138–139	D	
	HE3	Human (NH)	Stool		5	4	2	1	1	9	1	6	5	A	
	P2	Pig	Colon		5	5	2	1	1	3	1	184	5	A	
<i>erm</i> (B-2)	VZ200	Pig	Carcass		5	3	2	1	1	9	1	140	5	A	
	K331	Broiler	Cloaca	F	1	5	2	1	6	6	7	9	9	B	
	HE2	Human (H)	Urine		1	7	1	5	1	1	1	18	D18–186	C1	
	AME4	Pig	Colon		1	15	1	1	1	2	1	121	S	C	
	HE35	Human (NH)	Stool		1	9	3	1	1	6	1	25	9	C	
	P10	Pig	Colon		1	3	3	1	1	2	1	32	S	C	
	K44	Broiler	Cloaca	B	1	5	2	1	24	14	39	197	S	B	
<i>erm</i> (B-3)	P8	Pig	Colon		5	4	2	1	1	9	1	6	5	A	
	K40	Broiler	Cloaca	B	1	5	2	1	7	6	7	194	9	B	
<i>erm</i> (B-4)	K413	Broiler	Cloaca	G	1	2	2	1	1	30	11	199	S	B	
	P7	Pig	Colon		5	5	2	1	1	9	1	5	5	A	
	AME129	Pig	Colon		5	5	2	1	1	9	1	5	5	A	
	AME61	Pig	Colon		1	29	2	1	12	6	6	143	S	B	
	HE47	Human (H)	Blood		1	9	2	1	1	6	1	26	9	B	
	SH48	Pig	Carcass		5	28	2	2	1	12	34	144	S	NA	
	WV235	Pig	Carcass		8	5	4	1	12	6	1	142	S	NA	
	LO122	Pig	Carcass		5	5	5	1	1	9	1	141	5	A	
	<i>erm</i> (B-5)	K283	Broiler	Cloaca	I	1	9	2	1	1	3	33	200	S	B
		K57	Broiler	Cloaca	A	1	5	2	1	6	6	7	9	9	B
		K61	Broiler	Cloaca	A	1	5	2	1	6	6	7	9	9	B
		P3	Pig	Colon		1	31	1	1	1	1	1	187	17	C1
		AME136	Pig	Colon		5	3	5	1	1	3	14	138	D138–139	D
		AME46	Pig	Colon		1	4	5	1	1	3	1	29	D29–123	B
		HE15	Human (NH)	Stool		1	2	3	1	1	26	1	136	S	C
HE29		Human (NH)	Stool		1	2	3	1	1	26	1	136	S	C	
HE41		Human (H)	Stool		5	5	2	1	1	9	20	185	5	A	
HE55		Human (NH)	Stool		1	4	5	1	9	3	20	183	S	B	
P5		Pig	Colon		1	5	5	1	1	3	1	123	D29–123	B	
HE43		Human (H)	Urine		1	7	2	5	1	1	1	186	D18–186	C1	
K180		Broiler	Cloaca	C	1	5	2	1	1	6	7	8	9	B	
AME30		Pig	Colon		5	4	2	1	1	9	1	6	5	A	
V7A		Pig	Colon		5	4	2	1	12	9	20	149	S	A	
<i>erm</i> (B-6)	P1	Pig	Colon		5	5	2	1	1	9	1	5	5	A	
	P9	Pig	Colon		5	5	2	1	1	9	1	5	5	A	
<i>erm</i> (B-7)	K106	Broiler	Cloaca	E	1	16	2	2	6	6	7	198	S	B	
	K176	Broiler	Cloaca	D	1	5	2	1	7	6	7	194	9	B	
	K311	Broiler	Cloaca	I	1	9	2	1	6	3	7	82	9	B	
	K318	Broiler	Cloaca	F	1	5	2	1	6	6	36	196	9	B	
	K29	Broiler	Cloaca	B	1	5	2	1	7	6	7	194	9	B	
	K396	Broiler	Cloaca	F	1	9	2	1	1	6	7	195	9	B	
	K431	Broiler	Cloaca	G	1	9	2	1	7	6	1	124	9	B	
	K351	Broiler	Cloaca	G	1	5	2	1	7	6	7	194	9	B	
	<i>erm</i> (B-9)	HE46	Human (H)	Sterile wound		1	1	2	1	1	1	1	16	17	C1
		HE10	Human (H)	Abdominal fluid		1	1	1	1	1	1	1	17	17	C1
		HE40	Human (NH)	Urine		1	1	1	1	1	1	1	17	17	C1
		HE42	Human (H)	Urine		1	1	1	1	1	1	33	181	17	C1
		HE44	Human (H)	Urine		1	1	2	1	1	1	1	16	17	C1
		HE45	Human (H)	Urine		1	1	2	1	1	1	1	16	17	C1
		HE48	Human (H)	Blood		1	1	1	1	1	1	1	17	17	C1
HE49		Human (H)	Urine		1	1	1	1	1	1	19	182	17	C1	
AME81		Pig	Colon		1	7	1	5	1	1	1	18	D18–186	C1	
<i>erm</i> (B-10)	P6	Pig	Colon		1	3	3	1	1	2	1	32	S	C	
	HE22	Human (H)	Wound fluid		1	7	1	1	1	1	11	19	S	C1	
	K294	Broiler	Cloaca	H	1	9	2	1	6	6	7	157	9	B	
<i>erm</i> (B-11)	P4	Pig	Colon		1	15	2	1	12	6	1	49	S	B	

^a NA, not assigned; H, hospitalized; NH, nonhospitalized; ST, sequence type; CC, clonal complexes were identified using the eBURST algorithm (8) with stringent (6 of 7 shared alleles) group definition with 1,000 bootstrap replicates. Genogroup was determined by amplified fragment length polymorphism (AFLP) analysis (10).

contrast, CC5, with ST-5 as its presumed founder, could be classified as a pig-specific CC, since it consists of seven STs representing nine isolates from pigs and three isolates from humans (two NH and one H). The majority of isolates from

hospitalized patients grouped in CC17, with ST-17 as the presumed founder. Complex 17 has been recognized as a clinically relevant genetic complex harboring hospital outbreak and human clinical isolates from various countries on five continents

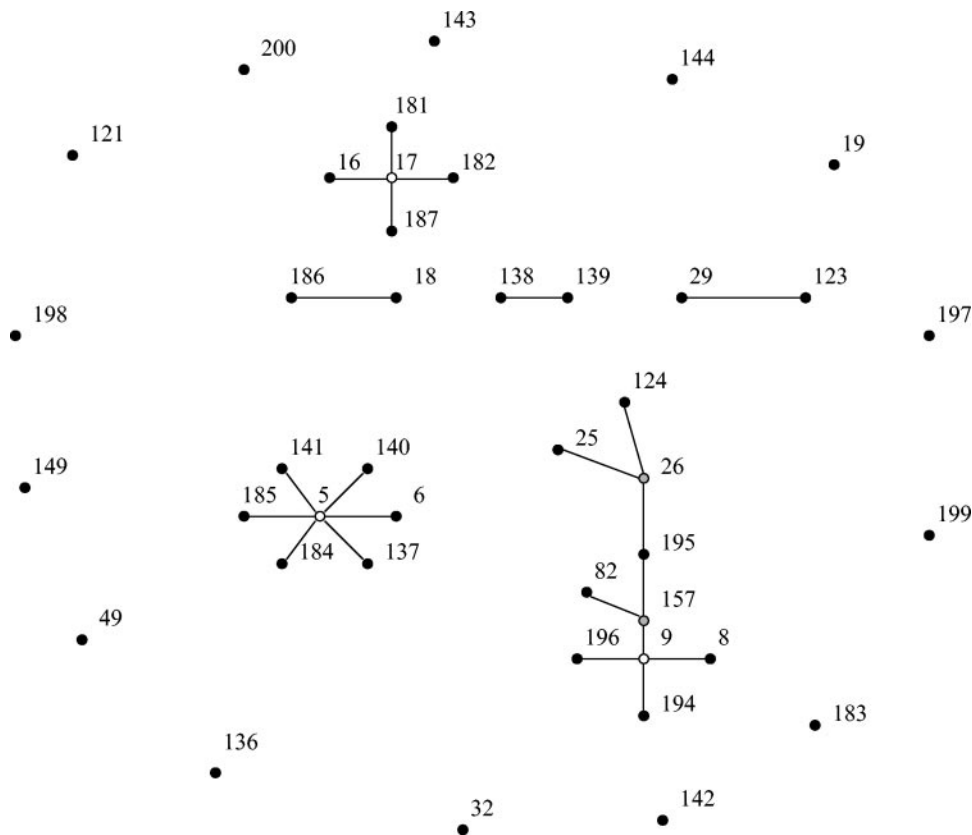


FIG. 1. Clustering of 42 sequence types, representing 59 isolates, with eBURST (8). This algorithm identifies the founder of a complex or genogroup of related sequence types and subsequent patterns of evolutionary descent. The primary founder of a complex, indicated with a white dot, is defined as the ST with the largest number of single-locus variants (SLVs). In larger complexes there may be secondary founders of additional lineages that have a number of SLVs of their own. These secondary founders are indicated in gray. Numbers correspond to ST numbers. The area of each circle corresponds to the number of isolates of the ST. All complexes (major and minor) and singletons (STs not belonging to any of the identified complexes) are shown.

(R. Willems et al., unpublished data). Also, one isolate from a nonhospitalized person and one isolate recovered from the colon of a pig grouped in CC17.

Grouping of the 59 isolates with 139 previously typed isolates (10) showed that all poultry isolates grouped in genogroup B, which predominantly contains poultry isolates, while 83% of the isolates recovered from hospitalized patients clustered in the 'epidemic' genogroup C1. Isolates from pigs and nonhospitalized persons were more dispersed over different genogroups (Table 1).

Sequence alignment and unweighted-pair group method with arithmetic mean clustering resulted in 11 allelic variations of the *erm*(B) gene, designated *erm*(B-1) through *erm*(B-11), which are more than 98.6% identical. The *erm*(B-3), *erm*(B-6), *erm*(B-7), and *erm*(B-11) sequences were found in one porcine isolate only. The *erm*(B-8) sequence was found in eight isolates, all originating from broilers, representing six different STs, of which five belonged to CC9, suggesting an evolutionary link between these seven broiler isolates (Fig. 1) (Table 1). The *erm*(B-1) and *erm*(B-9) sequences were detected in human isolates as well as in isolates originating from pigs. The *erm*(B-9) allele was predominantly found in isolates from hospitalized patients in CC17. *erm*(B-2), *erm*(B-4), *erm*(B-5), and *erm*(B-10) were found in genetically unrelated isolates from

humans, pigs, and broilers. Interestingly, in three more cases, a human- and animal-derived isolate carried an identical *erm*(B) gene and belonged to the same CC. The sequences *erm*(B-2), *erm*(B-4), *erm*(B-8), *erm*(B-9), and *erm*(B-10) were 100% identical to the *erm*(B) sequences in GenBank entries of *E. faecalis* plasmid pAM β -1 (Y00116), *E. faecalis* plasmid pRE25 (X92945), a genetic element containing *erm*(B) and *vat*(E) (AF242872), *E. faecium* plasmid pUW786 (AF516335), and transposon Tn917 (M11180), respectively (2, 13, 24, 26, 30).

The kanamycin resistance gene *aphA-3* was detected in 18 human, 10 porcine, and 3 broiler isolates. All isolates that carried the *erm*(B-9) allele contained the *aphA-3* gene. The glycopeptide resistance gene *vanA* was found in 13 human and 2 broiler isolates. This gene was detected in the four human isolates (HE42, HE44, HE45, and HE46) containing the *erm*(B-9) allele. Three of these isolates belonged to the same clone (ST-16) and all belonged to the same CC17. In 10 broiler isolates, including all isolates containing the *erm*(B-8) allele, the *erm*(B) gene was linked with the *vat*(E) gene. Such linkage was not detected in the other isolates.

MLST of a heterogeneous set of animal- and human-derived *E. faecium* isolates revealed that isolates from pigs and nonhospitalized humans are genetically more diverse than isolates

from broilers and hospitalized humans. These results are in agreement with other studies (3, 32).

Similarly, there is also a greater homology among the broiler *erm(B)* sequences, whereas more heterogeneity is seen among the porcine *erm(B)* sequences. In the eight broiler isolates containing the *erm(B-8)* allele, the *erm(B)* gene was linked with the *vat(E)* gene, confirming the wide distribution of this link among *E. faecium* poultry isolates found in other studies (13, 29). In contrast to Werner et al. (29), this link was not found among isolates from humans.

Although no porcine, broiler, and human strains with identical STs as well as identical *erm(B)* sequences were detected, the clustering of animal and human strains in four cases with identical *erm(B)* genes in clonal complexes indicates that these animal and human strains originate from the same founder, which may suggest an epidemiological link between these isolates. The human isolate HE3 and the porcine isolate VZ200 are single-locus variants (differing in one locus only) and contain an identical *erm(B)* gene. This might indicate a direct transmission of a resistant isolate between animals and humans and is in agreement with other studies (6, 28).

Several human isolates tested in the present studies belonged to the same clone. This might indicate intrahospital spread of these strains because the human isolates were collected in the same hospital. Also, two porcine isolates and three broiler isolates belonged to one clone. This is most probably due to interfarm spread, since these isolates originated from different farms. Broiler isolates K57 and K61 belong to the same clone and originated from the same farm, indicating clonal spread within a flock.

One porcine isolate (P3) belonged to CC17 that thus far contained no animal isolates. This may indicate a spread of a human strain to a pig. A human-to-animal transmission has also been described by Seguin et al. (25).

Within the three major complexes and two of the minor complexes, different *erm(B)* sequences were detected. In several cases, these alleles were identical to a described *erm(B)* sequence carried by a conjugative plasmid or transposon. This suggests that these strains may have acquired the *erm(B)* gene from different sources by horizontal transfer. This is in agreement with Simjee et al. and Descheemaeker et al. (6, 27).

In addition, identical *erm(B)* alleles were found in porcine, broiler, and human isolates belonging to different complexes. This also points towards horizontal transfer of the *erm(B)* gene in this case between genetically different *E. faecium* isolates from pigs, broilers, and humans or the existence of a common reservoir of *erm(B)* genes. This is in agreement with previous studies (6, 11, 23, 31).

The majority of the isolates belonging to CC17 carried the *erm(B-9)* allele. This sequence is identical to the *erm(B)* gene sequence found by Werner et al. (30) in a multiresistance gene cluster linking *vanA*, *erm(B)*, and *aadE-sat4-aphA-3* on plasmid pUW786 in a clinical isolate of *E. faecium* (30). In four isolates containing the *erm(B-9)* allele, *vanA* as well as *aphA-3* were also detected, suggesting the presence of pUW786. This plasmid might be responsible for the spread of this *erm(B)* allele in strains belonging to closely related STs that circulate in hospitals. The presence of *erm(B-9)* in the remaining isolates might be due to other mobile elements containing this allele. One may speculate on the independent evolution of the

erm(B) gene through mutations after introduction in a hospital strain followed by horizontal spread of this allele between hospital strains. This is in agreement with Willems et al. (31).

In conclusion, identical *erm(B)* sequences were found in genetically linked but also in genetically unrelated isolates from animals and humans. Furthermore, different *erm(B)* alleles were found in genetically indistinguishable isolates. This might indicate that resistance exchange between animals and humans possibly is due to direct transmission of resistant *E. faecium* strains, but horizontal exchange of the *erm(B)* gene between *E. faecium* isolates from animals and humans or the existence of a common reservoir of *erm(B)* genes might be more important.

Nucleotide sequence accession numbers. The six alleles not described previously were deposited in GenBank under accession numbers AY827541 [*erm(B-1)*], AY827542 [*erm(B-3)*], AY827543 [*erm(B-5)*], AY827544 [*erm(B-6)*], AY827545 [*erm(B-7)*], and AY827546 [*erm(B-11)*].

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