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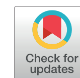
Molecular characterization of antibiotic resistant and potentially virulent enterococci isolated from swine farms and feed mills



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ABSTRACT

A total of 108 swine feed samples were collected from six feed mills and two farms and tested for enterococcal contamination. Nearly 43% of these samples were positive for enterococci. The mean ± SE concentration of enterococci in feed samples ranged from 2.0×10^1 to 7.3×10^3 CFU/g of feed. About 38% of processed feed mill samples were contaminated with enterococci compared to 59% of swine farm samples. A total of 208 enterococcal isolates were represented by *Enterococcus casseliflavus* (54.8% of total isolates), *E. gallinarum* (17.8%), *E. faecium* (17.8%), *E. hirae* (5.8%), and *E. faecalis* (3.8%). These isolates were phenotypically resistant to tetracycline (48.5%), erythromycin (14.4%), streptomycin (13.4%), kanamycin (11.5%), ciprofloxacin (10.0%), ampicillin (2.8%), and chloramphenicol (1.4%). All isolates were susceptible to vancomycin and gentamicin. Tetracycline resistance was encoded by *tetM* gene (52.8%), *tetO* (14.4%), *tetK* (1.0%), and *tetS* (0.5%), whereas *ermB* conferred erythromycin resistance in 10.6% of all isolates. Several isolates carried genes coding for virulence factors, including gelatinase (*gelE*; 18.2%), an enterococcal surface protein (*esp*; 2.4%), and cytolysin (*cylA*; 2.4%). Only *E. faecalis* was β-hemolytic (2.9%) and gelatinolytic (3.4%). The aggregation substance gene *asa1* was detected in 5 out of 8 *E. faecalis* isolates, of which four were phenotypically positive. The transposon Tn*916/1545* was detected in 11.5% of all isolates. Mating assays revealed that 7 out of 8 *E. faecalis* could transfer *tetM* gene, and rate of transfer ranged from 2.0×10^{-3} to 1.6×10^{-5}. The presence of antibiotic resistant and potentially virulent enterococci in swine farm samples and feed mill samples, though in low prevalence, raises concern and emphasizes the need for improved hygiene and quality standards on farms and in feed mills.

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1. Introduction

Animal feed plays a significant role in the food supply chain. There are growing concerns regarding contamination of animal feed before arrival at and while on the livestock farm leading to transfer of foodborne pathogens such as *Salmonella* spp., *Campylobacter* spp., *Yersinia* spp., *Listeria* spp. and enterohaemorrhagic *Escherichia coli*, from food animals to humans (Dorn et al., 1975; Teuber, 1999; Bailar and Travers, 2002; Crump et al., 2002; Angulo et al., 2004; Hawkes and Ruel, 2006; Lester et al., 2006; Maciorowski et al., 2006; da Costa et al., 2007; EFSA, 2008). Animal feed can potentially become contaminated with food-borne pathogenic bacteria at several points throughout the feed production, including the primary production of feed ingredients, milling, mixing, extrusion, storage, and transportation (Cox et al., 1983; Hofacre et al., 2001; Kidd et al., 2002; Myint et al., 2007; Sapkota et al., 2007). Additionally, the use of a wide range of antimicrobial drugs representing all major classes of clinically important antimicrobials, from penicillin to third-generation cephalosporin compounds, in food animal production in the United States and many other countries (Silbergeld et al., 2008) has led to the emergence of antibiotic resistant strains of *Enterococcus* in food animals on farms (Aarestrup et al., 2000; Garcia-Migura et al., 2005; Ahmad et al., 2011; Novais et al., 2013). Therefore, the ingredients used in animal feed, and hygienic conditions of the processing environment and storage facilities are fundamentally important in terms of both the quality of the resulting food products and the potential animal or human health impacts associated with the animal-based food production systems (Crump et al., 2002; Maciorowski et al., 2006; da Costa et al., 2007; Sapkota

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et al., 2007).

Enterococci traditionally have been considered to be of relatively low virulence in healthy individuals, but several species of enterococci have gained prominence in the last decade as the third leading cause of nosocomial infections in humans because of their resistance to several antibiotics, presence of virulence factors, and presence of an efficient horizontal gene transfer system (Coque et al., 1998; Gilmore, 2002; Gilmore et al., 2013). Enterococcal contamination of feed and feed ingredients by antibiotic-resistant *Enterococcus* species (de Costa et al., 2007; Ge et al., 2013), including vancomycin resistant *E. faecium*, has been reported (Schwalbe et al., 1999). There are increasing concerns of transfer of antibiotic resistant enterococcal strains from animal feed to humans through the food chain (Donabedian et al., 2003; Lester et al., 2006; Johnson et al., 2007; Hammerum et al., 2010). Additionally, the activity and by-products (fecal material) of stored-product insects, birds, and rodents in the feed environment may increase the chance of pathogenic bacterial contamination (Daniels et al., 2003; Channaiah et al., 2010a).

In our previous studies, we demonstrated that stored-product insects such as the red flour beetle, *Tribolium castaneum* (Herbst); confused flour beetle, *Tribolium confusum* Jacquelin du Val; warehouse beetle, *Trogoderma variable* Ballion; rusty grain beetle, *Cryptolestes ferrugineus* (Stephens); lesser grain borer, *Rhyzopertha dominica* (F.); drugstore beetle, *Stegobium paniceum* (L.); darkling beetle, *Tenebrio molitor* L; foreign grain beetle, *Ahasverus advena* (Waltl), and maize weevil, *Sitophilus zeamais* (Motschulsky), inhabiting United States feed mills carry antibiotic resistant and potentially virulent *Enterococcus* species in their gut (Larson et al., 2008; Channaiah et al., 2010a). Furthermore, we demonstrated that *T. castaneum* adults can act as potential vectors of antibiotic-resistant enterococci within the feed manufacturing environment, stressing the importance of proper pest management practices to reduce contamination of animal feed in feed mill environments (Channaiah et al., 2010b). However, it was unclear how insects acquire these enterococcal isolates. Therefore, to better understand the ecology of enterococci associated with animal feed, in the present investigation, feed samples collected from feed mills and swine farms were analyzed for enterococcal contamination to characterize their antibiotic resistance profiles. Specific research objectives of this investigation were to determine the prevalence, concentration, and diversity of antibiotic resistant and potentially virulent enterococci associated with animal feed. Additionally, the presence and transfer of mobile genetic elements in enterococci were examined.

2. Materials and methods

2.1. Collection of feed samples

Feed samples were collected from six feed mills and two confined swine facilities located in four Midwestern United States (Kansas-KS, Indiana-IN, Iowa-IA, and Wisconsin-WI). A total of 108 feed samples (67 from feed mills and 41 from swine farms) were collected over a period of five months. Feed samples (250 g) from mills included raw materials as well as various processed fractions and finished feed. Samples of feed (250 g) from storage facilities were collected from swine farms. The complete list of feed samples collected in this study is shown in Table 1. All samples were collected in a sterile zipper-sealed plastic bag, labeled, and transported in a cooler to the laboratory for microbial analyses.

2.2. Isolation, enumeration, and identification of enterococci

The feed sample in each sterile plastic bag was mixed manually for 10 min to ensure thorough mixing of feed materials, and a representative sample (10 g) was suspended in 100 ml of phosphate buffered saline (PBS) (pH 7.2; MP Biomedicals, Santa Ana, California, USA), vortexed for 10 min and then dilution-plated on m-Enterococcus agar (Difco Laboratories, Franklin Lakes, New Jersey, USA). Following incubation at 37 °C for 48 h, the colony forming units (CFU) were counted to determine the enterococcal concentration per gram of feed. Morphologically different presumptive enterococcal colonies were cultured on trypticase soy agar (TSA) (Difco Laboratories) and confirmed at the genus level by presumptive esculin hydrolysis test using Enterococcosel broth (Difco Laboratories). Enterococcal species were identified by multiplex Polymerase Chain Reaction (PCR) using species-specific primer sets for *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* (Kariyama et al., 2000). The strains used as positive and negative controls have been reported previously (Macovei and Zurek, 2006). *Enterococcus hirae* was identified by single PCR as described by Arias et al. (2006) and *E. hirae* ATCC 8043 was used as a positive control. The remaining unidentified isolates were identified by amplification and sequencing the manganese-dependent superoxide dismutase gene *sodA* (Poyart et al., 2000).

2.3. Phenotypic and genotypic screening of enterococci for antibiotic resistance

For antibiotic susceptibility screening, antibiotics were selected based on their use in animal agriculture as well as in clinical treatments. Antibiotic susceptibility was determined for all identified enterococcal isolates by the disk-diffusion assay on Mueller-Hinton agar (Difco Laboratories) using seven different antibiotics on separate paper disks at the prescribed standard amounts: ampicillin (10 μg), ciprofloxacin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), vancomycin (30 μg), and gentamicin (120 μg). Resistance to streptomycin (2000 μg/ml) and kanamycin (2000 μg/ml) were assessed by agar dilution technique on brain heart infusion (BHI; Difco Laboratories) agar. The disk-diffusion test is conducted by growing enterococcal lawn on the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Seven different paper disks with fixed concentration, were placed on the inoculated agar surface and plates were incubated for 16–24 h at 35 °C, after which the zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter, and interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) (CLSI, 2008, 2010). Routine quality control of antibiotic disks was performed using the *E. faecalis* ATCC 19433 strain.

Single and multiplex PCR was performed to screen all identified isolates for tetracycline and erythromycin resistance genes. The group I multiplex reaction included the *tetA, tetC,* and *tetQ* genes, while the group II multiplex reaction included the *tetM, tetS, tetK,* and *tetO* genes (Ng et al., 2001; Villedieu et al., 2003). Single PCRs were used to screen *tetW* (Aminov et al., 2001) and *ermB* (Sutcliffe et al., 1996). The PCR reaction and conditions were described previously by Macovei and Zurek (2006).

2.4. Genotypic and phenotypic screening of enterococci for virulence determinants

Enterococci possess several virulence factors such as enterococcal surface protein (*esp*), cytolysin activity (*cylA*), and gelatinase activity (*gelE*), encoded by transposons or mobile genetic elements. Enterococcal isolates were screened for the presence of virulence factors and transposons that can be ascribed to their roles in pathogenesis. Multiplex PCR was performed to screen all identified

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