Title: Towards controlling antimicrobial resistance in swine production systems: harnessing the paradoxical effects of micro-minerals and feed-grade antimicrobials on resistance in enteric bacteria - **NPB # 10-121.**

Revised

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Industry Summary:

The present study was aimed at determining the effects of supplemental feed grade antimicrobials on the selection and co-selection of copper resistance among enteric bacteria in post-weaning pigs. In our earlier studies, we showed that increased supplementation of copper selects for copper resistance among fecal *Enterococcus* spp. of piglets in the US and also co-selects for tetracycline and erythromycin traits co-located on the same genetic element (a very large plasmid). This represents a threat to the US animal agriculture since several of these bacteria have a high propensity for transferring their resistance determinants to other enteric bacterial flora (both commensal and pathogenic bacteria). To further these findings, we included in our present study both feed grade tylosin and chlortetracycline (commonly used antibiotics during the post-nursery stage) alone and also in combination with copper as per NRC guidelines. The study was conducted in a segregated early weaning facility consisting of two separate barns of 40 pens each.

Our study demonstrated that the supplementation of antibiotics in combination with copper does increase the prevalence of copper resistant enterococci when compared to feeding copper or antibiotics by themselves. All copper-resistant enterococcal isolates were also phenotypically and genotypically resistant to erythromycin and tetracycline (though the reverse was not always the case). It remains unclear at this time as to which practices producers should pursue to minimize selection for copper resistance among enterococci in commercial production settings. However, given the extremely rare presence of vancomycin resistant enterococci in U.S. swine production, it seems unlikely that co-selection of such resistance by copper, tylosin, or chlortetracycline will occur in the near future.

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Scientific Abstract:

Copper sulfate is often used as a growth promoter in swine production. Earlier work from our lab (Amachawadi et al., 2011) has illustrated the presence of the transferable copper resistance (*tcrB*) gene among swine enterococci and its co-location with *erm*(B) and *tet*(M) genes, coding for macrolide and tetracycline resistance, respectively, on the same transferable plasmid. The present study was undertaken to determine the effects of single or multiple feed grade antimicrobials on selection and co-selection of *tcrB*-positive enterococci in piglets. The study consisted of 240 weaned piglets, housed in groups of 5 animals per pen (n=48 pens). The pens were randomly allocated to six treatments (8 pens per treatment), arranged as an incomplete factorial design, comprising basal diets supplemented with none (control), copper (Cu), chlortetracycline (CTC), tylosin (Tyl), copper and tylosin (CuTyl), or copper and chlortetracycline (CuCTC). No combinations included both CTC and Tyl since this was not an

FDA-approved combination. The treatment phase was for 4 weeks followed by a washout phase for two weeks. Fecal samples were collected on days 0, 7, 14, 21, 28, and 35. All the enterococcal isolates were tested for the presence of *tcrB* gene by PCR. An equal number of *tcrB*-positive and matched-negative isolates (by pen, date, and treatment) were also tested for both *erm*(B) and *tet*(M) genes. A total of 372 enterococcal isolates were positive for the *tcrB* gene with an overall prevalence of 14.4%. The prevalence of *tcrB*-positive enterococci in each treatment group was: control (47/432; 10.8%), Cu (52/432; 12.0%), CTC (79/432; 18.3%), Tyl (51/432; 11.8%), CuCTC (75/432; 17.4%), and CuTyl (68/432; 15.7%). The *tcrB*-positive isolates had a mean copper MIC of 17.8 mM, compared to *tcrB*-negative isolates with an average MIC of 6.6 mM. Non-parametric analysis (log-rank test) of median and 90th percentile MIC values revealed a similar conclusion. All the *tcrB*-positive and matched-negative isolates also carried both *erm*(B) and *tet*(M) genes (conferring phenotypic resistance to erythromycin and tetracycline, respectively). The supplementation of additional antimicrobials had an additive effect beyond what would have been expected as a simple substitution and resulted in higher prevalence of the *tcrB* gene when compared to copper supplementation alone. Further studies are being undertaken to study both the phenotypic and genotypic differences among other enterococcal and *E. coli* isolates derived from the present study.

Introduction:

Animal agriculture has been blamed both for the emergence and the wide-spread dissemination of antibiotic-resistant bacteria world-wide (Aarestrup et al., 2008). Because of concerns about resistance, the use of antibiotics at subtherapeutic concentrations has been reduced or prohibited in countries of European Union (Threlfall et al., 2000; van den Bogaard et al., 2000). There is, therefore, an urgent need for interventions that will reduce antibiotic resistance at a faster pace. While advocates in Europe can claim some success in reducing levels of resistance in agricultural species (e.g., to vancomycin; by banning avoparcin use in food animals and poultry), in many cases antibiotic resistance trends have not shown the expected decreases since the ban (e.g., tylosin, and resistance macrolides; tetracycline resistance), especially with respect to those enteric bacteria harboring multiple antimicrobial-resistance determinants. This is likely due to the involvement of complex phenomena in the development of antimicrobial resistance. If effective, our proposed interventions in swine production systems will effectively reduce widespread bacterial expression of antibiotic resistance in a matter of weeks or months, instead of years.

Commensal bacteria such as *Escherichia coli* and *Enterococcus* spp. colonize the gastrointestinal tract of all warm-blooded animals and are present throughout multiple hosts and environments (Winfield and Groisman, 2003). There is a pressing need to better understand those complexities involving emergence, dissemination, propagation, and maintenance of antimicrobial resistance among enteric bacteria, especially *E. coli* and *Enterococcus* spp. In addition, these commensals can be pathogenic in certain agricultural situations (e.g., very young piglets and weanlings; see below), and in humans through either nosocomial infections (e.g., in hospital settings), or community-based extra-intestinal infections (e.g., urinary tract infections) (Collignon, 2002).

Escherichia coli primarily affect young piglets. These bacteria are the main cause of neonatal scours and also septicemia during first 14 days after weaning (Aarestrup et al., 2008). A very high frequency of resistance to commonly used antimicrobials can be found in some countries (though not consistently in others) and the antimicrobial susceptibility of *E. coli* can likewise be difficult to predict (Aarestrup et al., 2008). Generally, disease severity can be mitigated via the addition of antimicrobials such as tetracycline and tylosin, and also via heavy metals (copper and zinc) in the weanling diet. However, the final choice of antimicrobial to be used in the treatment and prevention of infection differs greatly due to the varying range of susceptibility among *E. coli* strains. There is evidence that upwards of 60-70% of the *E. coli* strains in modern swine production systems (including in organic and other antibiotic-free operations) are resistant to tetracycline (Bunner et al., 2007). The resistance patterns and dynamics can vary greatly among hosts and across different geographic locations.

Like *E. coli, Enterococcus* spp. are extremely versatile bacteria. Like other gut bacteria, some of the strains of *Enterococcus* are employed in the food manufacturing industry, or as probiotics (Facklam, et al., 2002; Simjee, et al., 2006). However, enterococci can also cause infections (Arias and Murray, 2008), particularly in hospital settings. Enterococci are intrinsically resistant to a broad range of antimicrobial agents and have evolved and acquired resistance to many of these by acquisition of plasmids or transposons from gram-negative and gram-positive bacteria (Kak and Chow, 2002). Thus, enterococci have emerged as important nosocomial pathogens (Malani et al., 2002). Enterococci are usually ranked second or third among the bacteria isolated from hospitalized patients (Kayser, 2003; Schaberg et al., 1991). Therefore, the public health importance of enterococci is related to the propensity of these organisms to participate in the horizontal transfer of the antimicrobials and virulence genes.

Copper, as copper sulfate, is sometimes supplemented in swine diets at concentrations of 100-250 ppm in order to reduce mortality and morbidity associated with bacterial enteric infections, particularly in piglets, and for growth promotion purposes (NRC, 1998). Perhaps incidentally, it has also been shown to enhance growth and efficiency of gain. Acquired copper resistance gene, designated as transferable copper resistance gene or *tcrB,* that confers copper resistance has been identified in *E. faecium, E. faecalis, E. gallinarum*, *E. casseliflavus*, and *E. mundtii* (Hasman and Aarestrup, 2002; Hasman et al., 2006). Interestingly, the plasmid also routinely carried the genes, *erm*(B),and *vanA,* that encode resistance to macrolides, and glycopeptides, respectively (Hasman and Aarestrup, 2002; Hasman et al., 2006), suggesting a potential linkage of copper resistance to antibiotic resistance.

Objectives:

- Investigate the prevalence of transferable copper resistance (*tcr*B) gene and its genetic linkages with macrolide and tetracycline resistance among commensal enteric bacteria of commercial pigs.

- Investigate the effects of copper supplementation on the prevalence of macrolide and tetracycline resistance phenotypes and genotypes, and their genetic linkages with *tcrB*, among fecal *Enterococcus* spp. and *Escherichia coli* of commercial pigs.
- Investigate the effect of supplementing swine diets with tylosin or chlortetracycline on the fecal prevalence of *tcrB*, among fecal *Enterococcus* spp. and *E. coli* of commercial pigs.
- Investigate fully the epidemiology and ecology of antimicrobial resistance among populations of fecal *Enterococcus* spp. and *E. coli* of commercial pigs; specifically, paradoxical changes to antibiotic and antimicrobial resistance prevalence that directly related to copper, tylosin, and chlortetracycline supplementation (and FDA permitted combinations thereof).

Materials & Methods:

a) **Animal trial and Experiments**: The animal trial was conducted from 2/9/2011 to 3/29/2011 at the segregated early weaning (SEW) facility of Kansas State University. The SEW facility has two metal buildings (south and north barns) with 40 pens each. We received weaned piglets on 2/9/2011 and randomized them by weight and allowed them to acclimatize to their surroundings and diet. All piglets received normal diet as per NRC recommendations. Following the brief period of acclimatization, we started the pigs on their medicated treatment diets (per random allocation). The treatment phase was continued for 4 weeks followed by a washout phase for another two weeks. The fecal samples were collected on days 0, 7, 14, 21, 28, and 35. The piglets were assigned randomly to six treatment groups by dividing each barn into 4 different quadrants. In total, we used 8 quadrants or replicates of treatment distributed equally among the barns. We collected fecal samples (rectal massage technique) from three piglets of each pen (of 5) during each sampling period, which gave us a total of 144 fecal samples for each sampling period.

b) Fecal sample processing and bacterial isolation: Unless otherwise mentioned all culture media used were from Difco (Becton and Dickson, Sparks, MD). The collected fecal samples were brought to the laboratory for processing on ice. A portion of each fecal sample was stored in 5 ml collection tubes both with and without glycerol for future use. The other portion of the fecal sample was used immediately for both *Escherichia coli* and *Enterococcus* sp. isolation. We used MacConkey and m-*Enterococcus* agar as the selective media for the isolation of *E. coli* and *Enterococcus* spp., respectively. The bacterial isolation was done by picking three distinct colonies from each plate (for both the bacteria) and re-streaking them onto blood agar plates. The colonies from the blood agar plates were subjected to basic biochemical tests like esculin hydrolysis for *Enterococcus* sp. and the indole test for *E. coli*, which aids in presumptive identification. We isolated 432 bacterial colonies (for each of *E. coli* and *Enterococcus* spp.) for each sampling period. After isolation and preliminary identification, we preserved these bacterial isolates on cryogenic beads (Key Scientific Products Inc. Stamford, TX) at -80°C for further use. In total, we have 2592 isolates of each of *E. coli* and *Enterococcus* sp. from our experiment.

c) PCR for the detection of the *tcrB*, *erm*(B), and *tet*(M) genes: The *tcrB* gene in enterococcal isolates was detected by the procedure described by Hasman *et al.* (2006). For DNA extraction, each isolate from the protect bead was streaked on a blood agar plate and a single colony was suspended in nuclease free water with Chelex® 100 Resin (Bio-Rad Laboratories, Hercules, CA) and boiled for 10 min. The primers (Table 1) for the PCR reaction were supplied by Integrated DNA Technologies (IDT, Coralville, IA). One of the two *tcrB*-positive *E. faecium* strain obtained from Denmark (7430162-6) served as a positive control. The primers (Table 1) and PCR conditions for detection of *erm* (*B*) and *tet* (*M*) genes were as per Jacob et al. (2008). *Enterococcus faecalis* MMH 594 and *E. coli* harboring plasmid pFD 310 (3) served as positive controls for *erm*(*B*) and *tet*(*M*), respectively.

d) Species identification of enterococci: Species identification of *tcrB*-positive and an equal number of *tcrB*-negative enterococcal isolates, randomly chosen from the control group, were performed by a multiplex PCR that identifies *E. faecium, E. faecalis, E. gallinarum* and *E. casseliflavus* (Jackson *et al.*, 2004) and by superoxide dismutase (*sodA)* gene sequence analysis (Poyart *et al.*, 2000). The DNA of the isolates was extracted as described above. Master mixes, primers (Table 1) and running conditions for the multiplex PCR were as described by Jackson et al. (2004). The ATCC strains of *E. faecium* (ATCC 19434), *E. faecalis* (ATCC 19433), *E. gallinarum* (ATCC 49579) and *E. casseliflavus* (ATCC 25788) served as positive controls. The primers (Table 1) and PCR conditions for *sodA* sequence analysis were as described by Poyart *et al.* (2000). The primers used were supplied by Invitrogen Life Technologies (Invitrogen, Carlsbad, CA).

e) Copper and antibiotic susceptibility determinations: Copper susceptibilities of enterococcal isolates were determined by agar dilution method (Hasman *et al.*, 2006). The *tcrB*-positive isolates (372), including the two strains from Denmark (7430162-6 and 7430275-4) and equal number of *tcrB*-negative strains selected matching treatments, pen, and date of collection were included. Mueller Hinton agars plates containing 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 or 40 mM of copper sulfate (Fischer Scientific, Fair Lawn, NJ), adjusted to pH 7.0, were used for copper susceptibility determinations. The plates, in duplicates, were spot inoculated with 20 µl of bacterial growth that was adjusted to McFarland turbidity standard no. 0.5. Plates were incubated for 48 h at 37°C to determine growth or no growth. The susceptibility determination was repeated with different inocula preparations. Minimum inhibitory concentrations of antibiotics were determined by micro-broth dilution method (CLSI, 2002). Antibiotics tested were erythromycin, and tetracycline (Sigma-Aldrich, St. Louis, MO). Stock solutions of antibiotics were prepared in sterile distilled water to obtain a concentration of 1,000 µg/ml based on potency of antibiotics. Antibiotics were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 and 0.098 µg/ml. The bacterial inocula were prepared by diluting (1:100) cultures grown in 10 ml Mueller Hinton II broth for 6 h and the concentration adjusted to 0.5 McFarland turbidity standards. The antimicrobial susceptibilities were performed in 96-well microtiter plates (Becton and Dickinson, Franklin Lakes, NJ). Plates were incubated at 37° C for 24 h and results were recorded as growth or no growth. Each concentration of the antibiotic was duplicated in the micro titer plate and MIC determinations were repeated with different inocula preparations.

f) **Statistical Analysis:** Data were analyzed using STATA SE (v. 12). The data was considered to be multilevel and longitudinal in nature since pens will be clustered within each treatment, animals will be clustered within pens, and within-animal dependency will exist because of repeated sampling of the same animals during the study period. The unbalanced factorial design with repeated measures and subsequent mixed model analysis will allow us to compare responses at specific times, over time, and the available interactions thereof. In addition, the main effects and interaction of the treatments was explored using random effects multi-level logistic regression model.

Results:

A total of 372 enterococcal isolates were positive for the *tcrB* gene with an overall prevalence of 14.4% ($P = 0.003$). The prevalence of *tcrB*-positive enterococci in each treatment group (Fig. 1) was: control (47/432; 10.8%), Cu (52/432; 12.0%), CTC (79/432; 18.3%), Tyl (51/432; 11.8%), CuCTC (75/432; 17.4%), and CuTyl (68/432; 15.7%). Both the treatments and week had a significant effect on the overall prevalence of *tcrB* gene ($P = 0.000$). However, among the treatments only CTC had a significant effect ($P = 0.040$) compared to Cu and Tyl. The categorization of week didn't affect the interpretation of the full model as it was also highly significant on the prevalence of the *tcrB* gene. Both the interaction term (Cu×CTC and Cu×Tyl) were not significant. The *tcrB*-positive isolates had a mean copper MIC of 17.8 mM, when compared to *tcrB*-negative isolate with an MIC of 6.6 mM (Fig. 2). Use on non-parametric analyses (i.e., MIC50 and MIC90: see Figure 3) revealed similar findings for median and 90th percentile, respectively. All the *tcrB*-positive and matched-negative isolates also carried both *erm*(B) and *tet*(M) genes with phenotypic resistance to erythromycin and tetracycline respectively. That is to say, 100% of the enterococci we recovered from swine in this study were resistant to both tetracycline and erythromycin no matter which treatment group, period, or whether copper-resistant or not.

Discussion:

The supplementation of additional antimicrobials had an additive effect beyond what would have been expected as a simple substitution and showed higher prevalence of *tcrB* gene when compared to copper alone. Further studies are being undertaken to study both the phenotypic and genotypic differences among other enterococcal isolates of the present study. Future work is directed towards:

1. We are in the process of further characterization of enterococcal isolates. Future work is mainly focused on understanding the emergence and dynamics of multidrug resistant enterococci, epidemiological studies on conjugative plasmids and their role in dissemination of antibiotic resistance, and paradoxical changes to antibiotic and antimicrobial resistance prevalence that directly related to copper, tylosin, and chlortetracycline supplementation.
2. Future work is also focused on the investigating the dynamics of transferable copper resistance among swine commensal *E. coli* isolates and their co-selection with other antibiotic resistance determinants. The role of plasmids and its epidemiology in the dissemination of antibiotic resistance that can be directly related to feed grade antimicrobials will be investigated.

List of figures

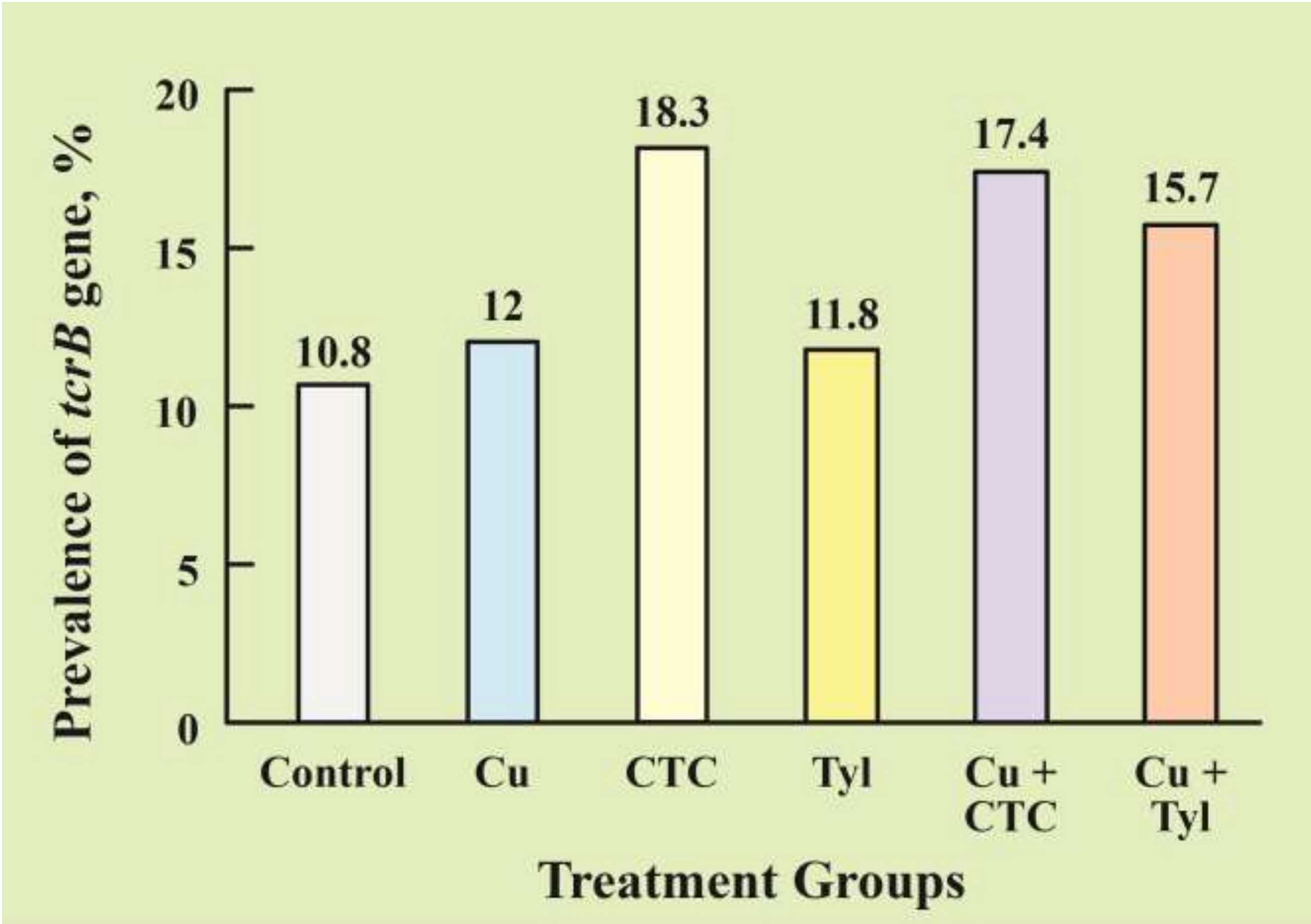

Fig. 1: The prevalence of *tcrB* gene (%) among different treatment groups

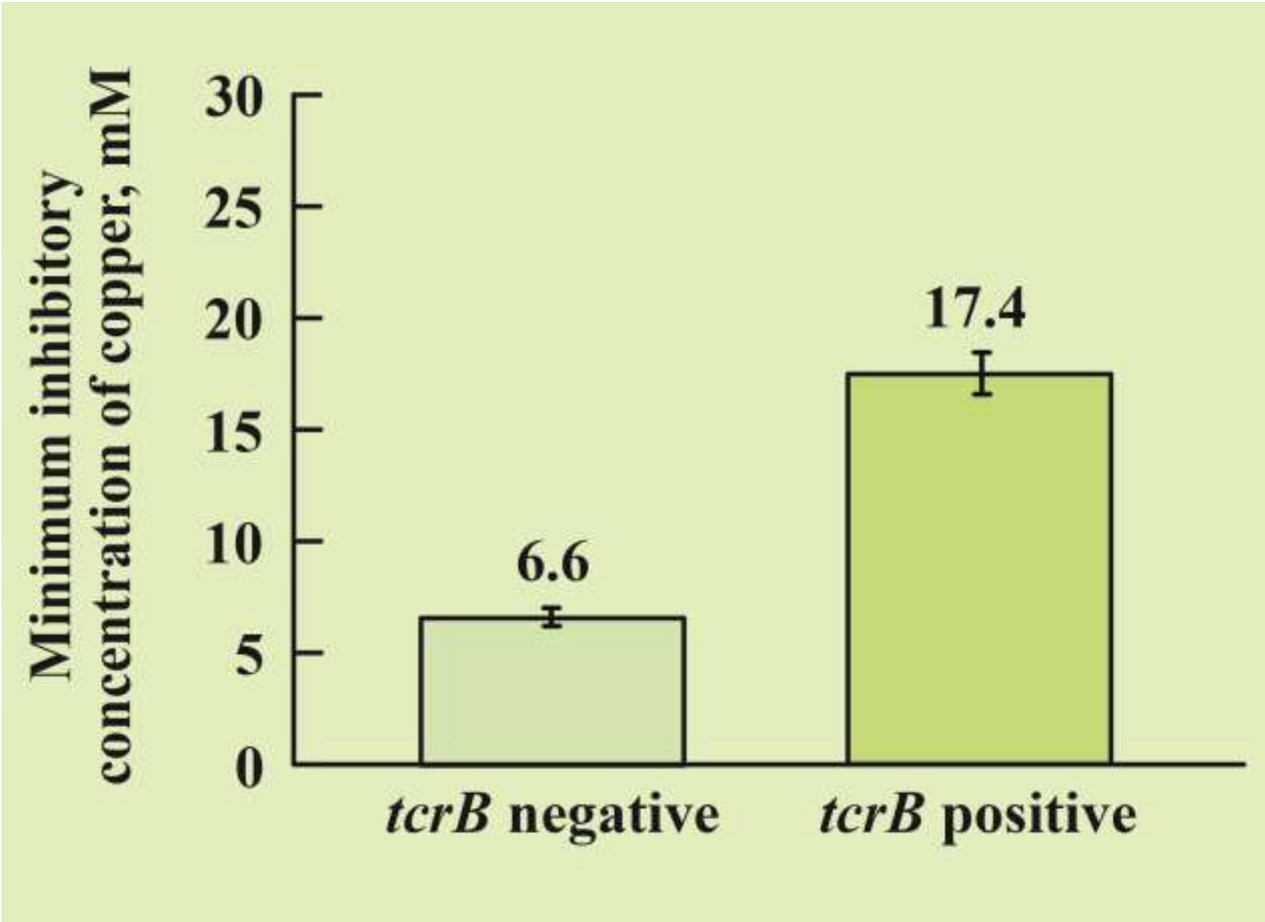

Fig. 2: Minimum Inhibitory Concentration of copper, mM for both *tcrB*-negative and –positive isolates.

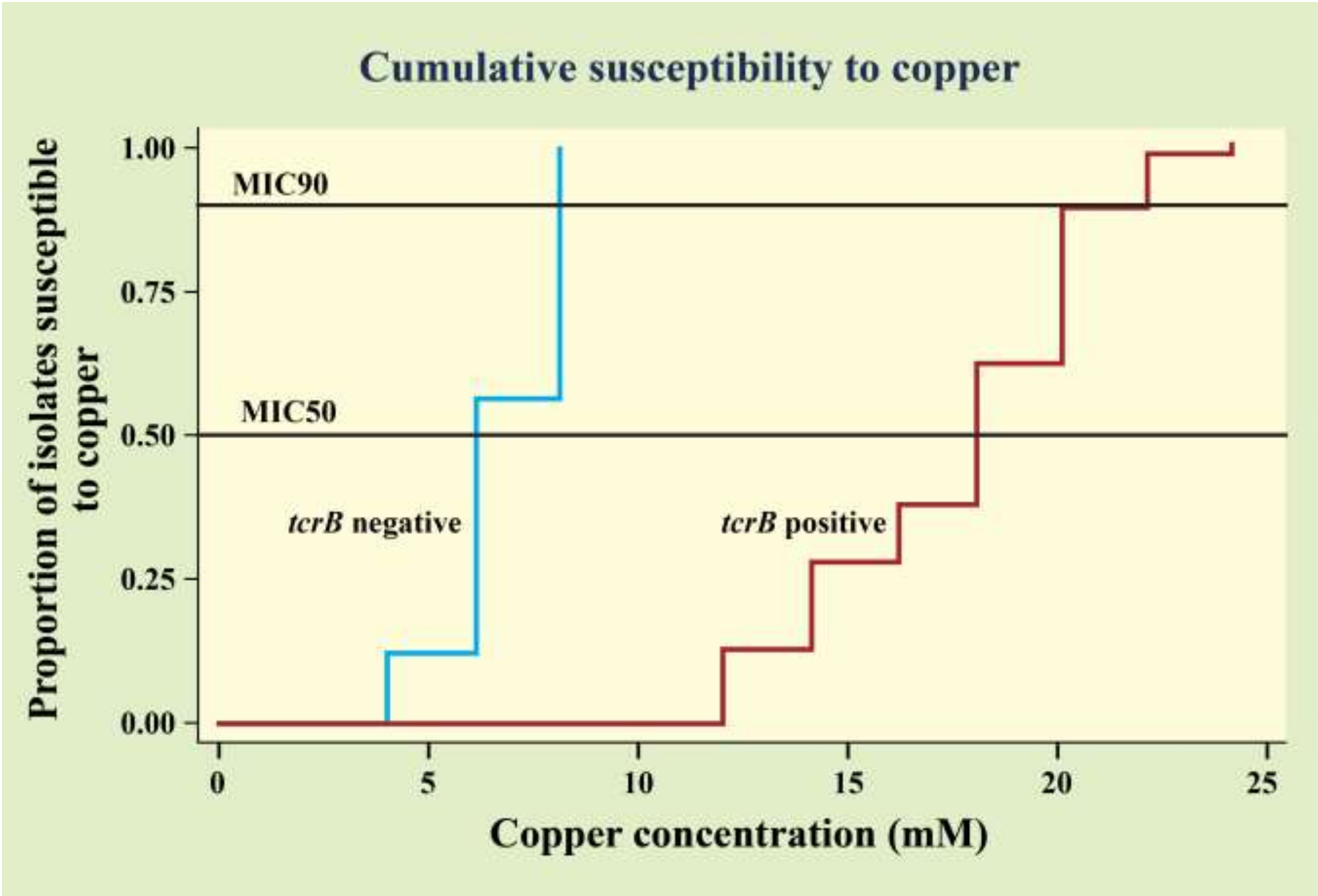

Fig. 3: Survival graph depicting proportion of isolates susceptible to copper (MIC 50 and MIC 90) for both *tcrB*-negative and –positive isolates.