Published in final edited form as:

Int J Antimicrob Agents. 2018 June ; 51(6): 897–904. doi:10.1016/j.ijantimicag.2018.02.003.

Repurposing Niclosamide for Intestinal Decolonization of Vancomycin-resistant Enterococci

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Abstract

Enterococci are commensal microorganisms present in the gastrointestinal (GI) tract of humans. Though normally innocuous to the host, strains of *Enterococcus* exhibiting resistance to vancomycin (VRE) have been associated with high rates of infection and mortality in immunocompromised patients. Decolonization of VRE represents a key strategy to curb infection in highly-susceptible patients. However, there is a dearth of decolonizing agents available clinically that are effective against VRE. The present study confirms that niclosamide, an anthelmintic drug, has potent antibacterial activity against clinical isolates of vancomycin-resistant *E. faecium* (minimum inhibitory concentration ranges from 1 to 8 µg/mL). *E. faecium* mutants exhibiting resistance to niclosamide could not be isolated even after multiple (10) serial passages. Based upon these promising *in vitro* results, and niclosamide's limited permeability across the gastrointestinal tract (when administered orally), niclosamide was evaluated in a VRE colonization-reduction mice model. Remarkably, niclosamide outperformed linezolid, an antibiotic used clinically to treat VRE infections. Niclosamide was as effective as ramoplanin in reducing the burden of vancomycin-resistant *E. faecium* in the feces, cecal content, and ileal content of infected mice after only eight days of treatment. Linezolid, in contrast, was unable to decrease the burden of VRE in the intestinal tract of mice. The results obtained indicate niclosamide warrants further evaluation as a novel decolonizing agent to suppress VRE infections.

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Competing Interests: No

Ethical Approval: All animal housing and experiments were reviewed, approved, and performed under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

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Keywords

Antibiotics; anthelmintic; decolonization; repurposing; resistance; salicylanilide

1. Introduction

Vancomycin-resistant enterococci (VRE), in particular species of *Enterococcus faecium* and *E. faecalis*, are a leading source of healthcare-associated infections globally [1]. Patients hospitalized for extended periods are often susceptible to exposure and colonization by enterococci that can subsequently lead to bloodstream infections and urinary tract infections [2, 3]. VRE infections, in particular, are often resistant to treatment with numerous antibiotics and thus are associated with a higher rate of mortality than infections caused by vancomycin-sensitive strains [4–6]. As highlighted in a recent study by Jiang *et al.*, the mortality rate of patients in a Taiwanese hospital infected with VRE was more than 64%, in contrast to 39.4% for patients infected with vancomycin-sensitive enterococci [7]. Moreover, the cost to treat patients infected with VRE nearly doubled in comparison to antibiotic treatment administered to patients infected with vancomycin-sensitive enterococci [7]. Therefore, suppressing VRE colonization in patients susceptible to infection represents a promising healthcare strategy.

Decolonization of VRE from the gastrointestinal (GI) tract is critical in certain patient populations, including those undergoing solid-organ (particularly liver) transplants [8] and patients in intensive care units [9], as VRE colonization has been linked to a higher risk for infection and death [9, 10]. However, decolonization of VRE is challenging. Antibiotics traditionally used to treat VRE infections are often ineffective as decolonizing agents in part due to rapid absorption from the gastrointestinal tract (linezolid) [11, 12]. Exceptions include the clinical molecule ramoplanin and the antibiotic bacitracin which are not absorbed from the GI tract after oral administration [13]. Ramoplanin temporarily reduced colonization of VRE in multiple studies [9, 14] and in nearly 90% of patients in a phase II clinical trial [12]. Similarly, bacitracin has been shown to be capable of temporarily reducing colonization of VRE from the intestinal tract of patients in multiple reports [15, 16]. However, VRE re-growth was observed in the intestinal tract of patients after cessation of treatment with both ramoplanin and bacitracin. Furthermore, resistance to bacitracin was observed in patients where this antibiotic was administered alone to decolonize VRE from the intestinal tract [9]. This highlights the challenges to using agents such as bacitracin and ramoplanin alone as decolonizing agents. An alternative approach to decolonization of VRE that has been investigated recently involves the use of fecal microbiota transplantation (FMT) [17, 18]. Though this approach successfully eradicated carriage of VRE in one human patient, it required three months to achieve this effect [18]. A second patient died three months after receiving FMT due to VRE infection, which suggests FMT alone may not be sufficient to decolonize VRE from the GI tract of certain patient populations [18]. The limited sample size (two patients) presented in Daviod *et al.*'s study suggest further investigation is needed to evaluate if FMT can be used as an alternative to decolonizing agents to curb VRE infection. This necessitates the continued search for novel agents that can successfully decolonize VRE from the intestinal tract.

Recently, the salicylanilide drug niclosamide was found to possess antibacterial activity against important Gram-positive bacterial pathogens, including vancomycin-resistant *E. faecium* [19, 20]. Niclosamide has been used clinically to treat tapeworm infections present in the intestinal tract of humans and has an excellent safety profile [21]. The anthelmintic drug possesses limited aqueous solubility, is poorly absorbed from the intestinal tract, and is rapidly metabolized by the liver after reaching systemic circulation [21]. While these features represent significant drawbacks to using niclosamide for treatment of systemic infections, these characteristics are highly advantageous for a decolonizing agent to reduce VRE carriage in the intestinal tract. Thus the main objective of this study was to examine niclosamide's potential to be successfully repurposed to decolonize VRE from the intestinal tract.

2. Material and methods

2.1. Media, strains, and reagents

Clinical isolates of vancomycin-resistant *E. faecium* and *E. faecalis* (Table 1) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and American Type Culture Collection (ATCC). Ampicillin (IBI Scientific, Peosta, IA, USA), closantel (Ark Pharm, Libertyville, IL, USA), gentamicin sulfate (Fisher Bioreagents, Fairlawn, NJ, USA), linezolid (Chem-Impex International Inc., Wood Dale, IL, USA), niclosamide (Cayman Chemicals, Ann Arbor, MI, USA), oxyclozanide (Sigma-Aldrich, St. Louis, MO, USA), rafoxanide (TCI America, Portland, OR, USA), ramoplanin (Sigma-Aldrich, St. Louis, MO, USA), and vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO, USA) were purchased from commercial vendors. Brain heart infusion (BHI), Tryptic soy broth (TSB), Tryptic soy agar (TSA) and enterococcosel agar were purchased from Becton, Dickinson and Company (Cockeysville, MD, USA). Phosphate buffered saline (PBS) was purchased from Corning (Manassas, VA, USA).

2.2. Antibacterial susceptibility analysis for salicylanilide drugs and control antibiotics

The minimum inhibitory concentration of salicylanilide drugs (closantel, niclosamide, oxyclozanide, and rafoxanide) and control antibiotics (linezolid, ramoplanin, and vancomycin) was determined using the broth microdilution assay following the guidelines outlined by the Clinical and Laboratory Standards Institute [22].

2.3. Time-kill assay for niclosamide, linezolid, and ramoplanin against vancomycin-resistant *E. faecium*

A time-kill assay was conducted for niclosamide, linezolid, and ramoplanin against *E. faecium* strain 503, following the same method described in a previous study [23]. Briefly, an overnight culture of *E. faecium* was diluted in BHI broth to reach a starting inoculum of 4.3×10^6 CFU/mL. Niclosamide and control antibiotics (tested at both $3 \times$ and $6 \times$ MIC), in triplicate, were incubated with the bacterial suspension for 24 hours. At the indicated time intervals, samples were taken, diluted in PBS, and an aliquot (4 μ L drop, two drops per replicate) from each dilution was plated onto BHI agar plates. Plates were incubated at 37 °C for 16 hours before counting colonies to determine viable CFU/mL.

2.4. Multi-step resistance selection for niclosamide and control antibiotics against vancomycin-resistant *E. faecium*

We investigated *E. faecium* strain 503's ability to develop resistance to niclosamide, linezolid, ramoplanin, and gentamicin using the multi-step resistance selection experiment (via the broth microdilution assay), as described in previous reports [23–25]. Microtiter plates containing the bacterial suspension ($\sim 5 \times 10^5$ CFU/mL) and niclosamide, gentamicin, linezolid, or ramoplanin (tested in triplicate) at different concentrations (ranging from 0.5 to 512 μ g/mL) were incubated at 37 °C for a minimum of 16 hours before the MIC was determined by visual inspection. Bacteria were serially passaged over 10 days, and the MIC was determined daily for each test agent. Resistance was classified as a greater than four-fold increase from the initial MIC [26].

2.5. *In vivo* assessment of niclosamide and control antibiotics in a VRE decolonization mice model

All animal housing and experiments were reviewed, approved, and performed under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Each group of mice was housed in an individually ventilated cage, and the bedding was changed regularly (every 2–3 days). In order to evaluate the ability of niclosamide and control antibiotics to decrease the burden of vancomycin-resistant *E. faecium* present in the GI tract of mice, the VRE-colonization reduction study described by Seiler *et al* [27] was utilized, with the following modifications. Briefly, female 12 week old C57BL/6 mice (Envigo, Indianapolis, IN) (n = 5 mice per group) were exposed to ampicillin (0.5 g/mL in drinking water) for seven days (day –7 to –1) in order to reduce the microbiota present in the GI. On day 0, ampicillin sensitization was stopped and mice were infected via oral gavage with 3.4×10^8 CFU/mL of *E. faecium* strain 503. Seven days post-infection (on day +7) each group of mice was treated via oral gavage with either 10 mg/kg of niclosamide, linezolid, or ramoplanin. One group of mice was left untreated (as a negative control). Mice were treated once daily for eight consecutive days. Fecal pellets (from each group) were freshly collected from mice at days 0, 3, 5 and 7 of the treatment period. One day after the eighth dose was administered, mice were humanely euthanized using CO₂ asphyxiation. The cecum and ileum were aseptically removed from each mouse. Fecal pellets, cecal content, and ileal content were diluted in PBS, plated on enterococcosel agar plates (supplemented with 8 μ g/ml vancomycin to select for VRE), and incubated at 37 °C for 48 hours to determine the bacterial CFU present in each sample.

3. Results

3.1. Susceptibility analysis of niclosamide and other salicylanilide drugs against clinical isolates of VRE

As highlighted earlier, Rajamuthiah *et al.*, were the first group to identify that niclosamide possesses antibacterial activity against VRE [20]. However, their study only screened niclosamide against a single strain of vancomycin-resistant *E. faecium* (strain E007, MIC = 0.25 μ g/mL) [20]. Thus we moved to evaluate niclosamide against a wider-panel of VRE

clinical isolates (Table 1) using the broth microdilution assay. As presented in Table 2, niclosamide inhibited growth of 15 clinical isolates of *E. faecium* at concentrations ranging from 1 µg/mL up to 8 µg/mL. This was similar to the activity observed for linezolid (MIC ranged from 0.5 to 16 µg/mL) and ramoplanin (MIC ranged from 0.25 to 4 µg/mL). In addition to niclosamide, there are three other salicylanilide drugs (closantel, rafoxanide, and oxyclozanide) used clinically; however their use is restricted to veterinary medicine. When these three drugs were evaluated against vancomycin-resistant *E. faecium*, closantel inhibited growth at concentrations that were equivalent to or one-fold higher than the MIC of niclosamide against the same strains. Rafoxanide and oxyclozanide were less active *in vitro* than niclosamide, inhibiting growth of *E. faecium* at concentrations that were up to seven-fold higher than the MIC of niclosamide against the same clinical isolates.

Interestingly, niclosamide was less effective against strains of vancomycin-resistant *E. faecalis* (MIC ranged from 32 to 128 µg/mL) indicating that *E. faecium* is more sensitive to niclosamide (Table 2). This was similar to results obtained for two other salicylanilide drugs (rafoxanide and oxyclozanide). However, closantel demonstrated the ability to inhibit growth of all clinical isolates of vancomycin-resistant *E. faecalis* examined, at concentrations ranging from 1 to 2 µg/mL, similar to both linezolid and ramoplanin. Given *E. faecium*'s higher susceptibility to niclosamide, we focused on this particular species for the remainder of the study.

3.2. Evaluation of niclosamide's antibacterial activity against *E. faecium* via a time-kill assay

After confirming niclosamide's antibacterial activity against vancomycin-resistant *E. faecium*, we examined whether the agent exhibited bacteriostatic or bactericidal activity. Niclosamide, linezolid, and ramoplanin were evaluated against *E. faecium* via a standard time-kill assay. As presented in Fig. 1, in the presence of niclosamide (both at 3 × and 6 × MIC), the bacterial CFU stayed constant over 24 hours, indicating the drug is bacteriostatic. This was similar to the pattern observed with linezolid, a known bacteriostatic agent against *E. faecium* [23]. Ramoplanin, in contrast, completely eradicated bacterial inoculum within two hours (at 6 × MIC) and six hours (at 3 × MIC).

3.3. Multi-step resistance selection of niclosamide against vancomycin-resistant *E. faecium*

A significant challenge of suppressing enterococcal infections is the pathogen has remarkable ability to develop resistance to antibacterial agents. Thus prior to evaluating niclosamide *in vivo* in an animal model, we investigated *E. faecium*'s ability to develop resistance to niclosamide using the multi-step resistance selection experiment. As depicted in Fig. 2, the MIC of niclosamide only increased one-fold over ten passages indicating *E. faecium* had not developed resistance to this drug. This was similar to results obtained for ramoplanin. No increase in the MIC for linezolid was observed. In contrast, *E. faecium* developed resistance rapidly to gentamicin as a seven-fold increase in MIC was observed after only the second passage.

3.4. *In vivo* examination of niclosamide and control antibiotics in a VRE-colonization reduction mouse model

The promising *in vitro* results obtained led us to evaluate niclosamide's ability to suppress the burden of vancomycin-resistant *E. faecium* *in vivo* using the VRE colonization-reduction mice model [27]. As presented in Fig. 3, niclosamide and ramoplanin were superior to linezolid in decreasing the burden of VRE in fecal samples collected from mice. After only three days of treatment, niclosamide reduced the burden of VRE in fecal samples by 2.1- $\log_{10}$, similar to ramoplanin (2.4- $\log_{10}$ reduction in VRE). Linezolid, in contrast, was unable to reduce the burden of VRE after three days of treatment. The presence of VRE continued to diminish resulting in a remarkable 3.1- $\log_{10}$ reduction in fecal samples of mice treated with niclosamide, after seven days of treatment. Mice treated with ramoplanin for seven days exhibited a 3.9- $\log_{10}$ reduction of VRE in fecal samples. Linezolid only generated a 0.8- $\log_{10}$ reduction in VRE CFU in fecal samples obtained from mice after seven days of treatment. The burden of VRE ($\sim 10^7$ CFU/gram feces) remained consistent in the control group (untreated mice) over seven days, indicating the decrease in VRE burden observed in mice receiving niclosamide, ramoplanin, or linezolid was due primarily to the treatment received (and not elimination of bacteria from the intestinal tract).

Similar to the fecal samples, niclosamide and ramoplanin generated significant reductions in VRE in the cecal and ileal contents of mice as compared to the untreated control group and mice treated with linezolid (Fig. 4). Niclosamide decreased the burden of VRE in the cecal content by 2.4- $\log_{10}$ and in the ileal content by 1.8- $\log_{10}$, relative to untreated mice. This was similar to results obtained for ramoplanin which decreased VRE in the cecal content by 3.3- $\log_{10}$ and in the ileal content by 2.6- $\log_{10}$, relative to untreated mice. Linezolid, in contrast, did not reduce the burden of VRE in either the cecal or ileal content.

4. Discussion

Vancomycin-resistant *Enterococcus* infections represent a significant challenge to healthcare providers given the limited number of effective antibiotics available to clinicians. VRE is responsible for 30% of all *Enterococcus* healthcare-associated infections (20,000 cases annually) and more than five percent of all deaths attributed to an antibiotic-resistant infection in the United States of America (USA) alone [28]. In addition to the USA, VRE infections have been identified in patients worldwide including in Europe, Latin America, and Asia [3]. Nearly 80% of all healthcare-associated VRE infections and half of all deaths attributed to VRE, in the USA, are caused by strains of *E. faecium* [28]. It is not surprising therefore that the World Health Organization recently categorized vancomycin-resistant *E. faecium* as one of twelve bacterial pathogens where new therapeutics and alternative strategies to combat infection are urgently needed [20]. Enterococci are commensal residents of the GI tract in humans; thus, decolonization of VRE from the intestinal tract represents an alternative strategy to quell infection in highly-susceptible individuals, such as patients undergoing solid organ (particularly liver) transplants. However, as highlighted earlier, antibiotics commonly used to treat VRE infections (such as linezolid) are typically ineffective as decolonizing agents. This necessitates the search for alternative approaches to finding novel decolonizing agents.

Ideally, a decolonizing agent for VRE should exhibit potent antibacterial activity, demonstrate reduced likelihood for VRE to develop resistance to this agent, exhibit limited permeability across the GI tract (so as to accumulate at a sufficient concentration at the target site), and possess limited toxicity to intestinal tissues. Niclosamide, an anthelmintic drug approved for use in humans in 1960, possesses all of these qualities. The toxicity profile of niclosamide has been well-characterized in humans and has been found to be safe with an LD₅₀ that exceeds 5000 mg/kg in rats [29]. In previous reports, no evidence of developmental toxicity, mutagenicity or carcinogenicity was observed in humans exposed to niclosamide, further corroborating that the drug has an excellent safety profile [30].

Interestingly, as discovered in this study, niclosamide is more active against strains of vancomycin-resistant *E. faecium* compared to *E. faecalis* (MIC ranged from 32 to 128 µg/mL). This species difference in susceptibility to niclosamide is similar to what has been reported for other antibiotics. For example, *E. faecium* is susceptible to clindamycin and quinupristin–dalfopristin, while certain strains of *E. faecalis* are resistant to these antibiotics [31]. Of note, closantel, another salicylanilide drug, was found to possess potent antibacterial activity against both vancomycin-resistant *E. faecalis* and *E. faecium*. Though closantel and niclosamide are structurally analogous, they differ in certain physicochemical properties. For example, there is a major difference in lipophilicity between closantel (clogP: 7.4) and niclosamide (clogP: 4.3). Additionally, closantel is twice as large (molecular weight: 663.07 g/mol) as niclosamide (molecular weight: 327.12 g/mol). Thus it is plausible that these structural differences may contribute to the difference in activity observed between these two drugs against *E. faecalis*, though further investigation is necessary. However, a challenge to utilizing closantel as a decolonizing agent in humans is the drug is only approved for use in veterinary medicine and is not recommended for use in humans [32]. Thus we chose to evaluate niclosamide further by focusing on its potential use as a decolonizing agent against vancomycin-resistant *E. faecium*.

Prior to evaluating niclosamide's effectiveness in a murine VRE-colonization reduction model, we examined the ability of vancomycin-resistant *E. faecium* to develop resistance to niclosamide. Enterococci are remarkably adaptive microorganisms capable of developing or acquiring resistance to antibacterial agents, including to newer antibiotics such as daptomycin [3, 33]. This was observed in our multi-step resistance selection experiment where *E. faecium* developed resistance to gentamicin (MIC increased by seven-fold) after only two passages. Though gentamicin is not used to treat VRE infections clinically, it has previously been evaluated as a decolonizing agent in combination with bacitracin [34]. No resistance to niclosamide was observed *in vitro* in the multi-step resistance study with *E. faecium* even after ten serial passages (only one-fold increase in MIC occurred).

The potent antibacterial activity coupled with the inability of *E. faecium* strain 503 to acquire resistance *in vitro* to niclosamide led us to evaluate the drug's effectiveness in a murine VRE-colonization reduction model. Niclosamide significantly reduced the burden of VRE by more than 2-log₁₀ in the feces and ilea of infected mice after only eight days of treatment. A point worth noting is niclosamide was administered to mice at a dose (10 mg/kg) that is within range of what is administered clinically to humans. The recommended dosing regimen for adult humans receiving niclosamide for treatment of parasitic infections

(*Hymenolepis nana* infection) is a single dose of 2000 mg niclosamide daily for seven days [35]. For an average adult weighing between 60 to 80 kg, this would translate to a dose of 25 – 33 mg/kg niclosamide, more than twice the dose used in our mice study. In a clinical trial that demonstrated niclosamide was effective in treating infections caused by *H. nana*, *Taenia saginata*, or *Diphyllobothrium latimi*, niclosamide was administered as a single dose of 2000 mg orally daily for 5 – 7 days in patients weighing over 56 kg. This translates to a dose equivalent to 35 mg/kg, which is more than three times higher than the dose used in our study [36]. Furthermore, niclosamide has even been administered to children at a dose of 80 mg/kg with no adverse toxicity reported [37]. This opens the door for future investigation to examine if increasing the size of the dose of niclosamide can lead to complete clearance of VRE from the GI tract in infected mice.

In the VRE-colonization reduction mice model, niclosamide proved significantly more effective than linezolid at reducing the burden of vancomycin-resistant *E. faecium* in the GI tract of infected mice. Though linezolid is used to treat VRE infections, it has not been proposed for use as a decolonizing agent. This is due in part to linezolid achieving a very low concentration in the stool of humans [38] and linezolid's propensity to promote overgrowth of VRE when a high inoculum of bacteria (10^8 CFU) is present [39]. Both niclosamide and linezolid exhibited bacteriostatic activity against VRE *in vitro* via a standard time-kill assay. However, niclosamide was superior to linezolid in reducing the burden of VRE *in vivo* in the intestinal tract of infected mice. This suggests that bacteriostatic agents, particularly those agents with limited ability to permeate across the GI tract, should not be excluded from consideration as decolonizing agents to prevent VRE infections. We suspect that the rapid absorption of linezolid from the GI tract limits its exposure time to VRE present in the intestinal tract. In contrast, niclosamide's limited absorption from the GI tract permits it to remain in the intestinal tract for an extended time period which appears to be sufficient to significantly decrease the burden of VRE present.

Remarkably, niclosamide was similar to ramoplanin, a potent bactericidal antibiotic *in vitro*, in its effectiveness in decreasing the burden of VRE in the intestinal tract of infected mice. Ramoplanin is not absorbed from the GI tract after oral administration, similar to niclosamide. Ramoplanin's potential application as a decolonizing agent has been validated in a phase III clinical trial as the drug was able to prevent bloodstream infections caused by VRE [12]. However, as stated earlier, one of the challenges reported with using ramoplanin alone has been re-growth of VRE after cessation of treatment [1, 40]. Of note, one of the advantages of niclosamide over ramoplanin is that niclosamide is an approved drug while ramoplanin still remains a clinical candidate. Using niclosamide may avoid side effects associated with traditional antibiotics and adverse events particularly on the gut microbiota. Indeed, the use of ramoplanin for instance, can increase the selective pressure and contribute to the selection of other multidrug-resistant organisms. Future studies will need to be conducted to determine if increasing the dose or prolonging treatment of niclosamide (beyond seven days) will ultimately lead to complete clearance of VRE from the GI tract of infected mice.

As noted in the introduction, fecal microbiota transplantation (FMT) has been recently investigated as an alternative approach to antibiotics to decolonize VRE from the GI tract. A

study by Mahieu *et al.* investigated the effect of FMT to decolonize VRE in mice [41]. After disruption of the resident microflora (via antibiotic treatment) and subsequent infection with vancomycin-resistant *E. faecium*, mice received FMT orally (200 µL of 50 mg/mL stool in saline) once daily for three days. Remarkably, seven days after the first FMT dose was administered, the burden of VRE in the fecal content of mice decreased by 8.2 log₁₀ [41]. However, re-growth of VRE was observed thereafter. This study indicates FMT alone may help significantly decrease VRE carriage in the GI tract during treatment; however, for complete eradication to occur, FMT may need to be combined with a decolonizing agent. Thus future investigation involving combination therapy using FMT and a decolonizing agent (such as niclosamide) may open a new avenue to eradicate VRE carriage from the GI tract.

Though niclosamide was effective in the VRE-colonization reduction mouse model, there are two limitations present in this study that would need to be addressed as part of a future investigation. First, we did not evaluate the potential for VRE re-growth in infected mice after cessation of treatment with niclosamide. As noted earlier, VRE expansion after cessation of treatment was a problem noted with ramoplanin and bacitracin when each was investigated as a potential decolonizing agent. A second limitation with this study is we did not evaluate the impact of niclosamide on the gut microflora. The delicate balance present between Gram-positive and Gram-negative bacteria that form the gut microbiota plays an important role in suppressing VRE colonization in the GI tract [3]. Hospitalized patients exposed to broad-spectrum antibiotics that diminished the presence of the Gram-negative microbiota resulted in the expansion of VRE in the GI tract (which may have led to subsequent infection) [3]. A previous report investigating ramoplanin as a decolonizing agent against VRE found that ramoplanin altered the anaerobic microbiota present in the GI tract (particularly affecting Gram-positive microorganisms) which resulted in the overgrowth of resistant Gram-negative bacilli (namely *Klebsiella pneumoniae* and *Enterobacteriaceae*) [14]. Thus a future investigation will need to evaluate niclosamide's impact on the gut microbiota to investigate if this drug has any deleterious impact.

In conclusion, the present study highlights that niclosamide has potent *in vitro* and *in vivo* activity against vancomycin-resistant *E. faecium* and warrants further investigation as a novel decolonizing agent to suppress VRE infection, particularly in high-risk (immunocompromised) patients.

Acknowledgments

The authors would like to thank Dr. Abdelrahman S. Mayhoub (Al-Azhar University, Cairo, Egypt) for valuable feedback regarding the structure-antibacterial activity relationship between niclosamide and other salicylanilide drugs.

Funding: This work was supported by the National Institutes of Health [grant number R01AI130186]. Haroon Mohammad is supported by a fellowship from the Purdue Institute for Drug Discovery.

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Highlights

- Niclosamide, an anthelmintic, is evaluated as a decolonizing agent for enterococci
- Niclosamide has antibacterial activity against *E. faecium* but not *E. faecalis*
- Vancomycin-resistant *E. faecium* (VRE) was unable to form resistance to niclosamide
- Niclosamide significantly reduced VRE shedding in feces of colonized mice
- Niclosamide significantly decreased VRE in the ceca and ilea of infected mice

Fig 1. Time-kill assay for niclosamide, linezolid, and ramoplanin (tested at 3 × MIC and 6 × MIC) against vancomycin-resistant *E. faecium*
VRE strain 503 was incubated with test agents and samples were collected over a 24-hour incubation period. DMSO, solvent for niclosamide and linezolid, served as the negative control. The error bars represent standard deviation values obtained from triplicate samples used for each agent studied.

Fig 2. Multi-step resistance selection of gentamicin, niclosamide, linezolid, and ramoplanin against vancomycin-resistant *E. faecium* strain 503

Bacteria were serially passaged with test agents for ten days and the broth microdilution assay was used to determine the minimum inhibitory concentration (MIC) of each test agent against VRE after each successive passage. A four-fold shift in the MIC is defined as resistance having formed to a test agent.

Fig 3. Burden of vancomycin-resistant *E. faecium* strain 503 in fecal content of infected mice
Fecal pellets were collected from mice treated with niclosamide (10 mg/kg), linezolid (10 mg/kg), or ramoplanin (10 mg/kg) once daily for eight days. Stool samples were collected before treatment (day 0), and on days 3, 5, and 7 after treatment and the viable CFU/g fecal content was determined. Data were analyzed via a two-way ANOVA with post-hoc Dunnet's multiple comparisons test. One asterisk (*) indicates significant difference ($P < 0.05$) between mice treated with linezolid, niclosamide, or ramoplanin compared to untreated mice. Two asterisks (**) indicates significant difference ($P < 0.05$) between mice treated with either niclosamide or ramoplanin compared to mice treated with linezolid.

Fig 4. Burden of vancomycin-resistant *E. faecium* strain 503 in infected mice (n = 5 mice per group) after seven days of treatment

a.) Log₁₀ CFU/g of VRE in cecal content of mice treated with niclosamide (10 mg/kg), linezolid (10 mg/kg), or ramoplanin (10 mg/kg) once daily for eight days. Data were analyzed via a two-way ANOVA with post-hoc Dunnet's multiple comparisons test. One asterisk (*) indicates significant difference ($P < 0.05$) between mice treated with either linezolid, niclosamide, or ramoplanin compared to untreated mice. Two asterisks (**) indicates significant difference ($P < 0.05$) between mice treated with either niclosamide or ramoplanin compared to mice treated with linezolid. b.) Log₁₀ CFU/g *E. faecium* strain 503 in mice ileal contents after eighth day of treatment. Data were analyzed via a two-way ANOVA with post-hoc Dunnet's multiple comparisons test. One asterisk (*) indicates significant difference ($P < 0.05$) between mice treated with either linezolid, niclosamide, or ramoplanin compared to untreated mice. Two asterisks (**) indicates significant difference ($P < 0.05$) between mice treated with either niclosamide or ramoplanin compared to mice treated with linezolid.

Table 1

Clinical isolates of vancomycin-resistant enterococci (VRE) utilized in this study.

VRE Strain	ID Number	Description
<i>E. faecium</i> , E0120	NR-31914	Isolated in 1995 from ascites fluid of a hospitalized patient in the Netherlands. Resistant to gentamicin and vancomycin.
<i>E. faecium</i> , ERV102	HM-968	Isolated from human oral sputum in Colombia (2006). Resistant to ampicillin, vancomycin, and displays high-level resistance to streptomycin.
<i>E. faecium</i> , E1071	NR-28978	Collected from a hospitalized person free of enterococcal infection in the Netherlands in 2000 during a surveillance program.
<i>E. faecium</i> , Patient #1-1	NR-31903	Isolated from the stool of a human patient prior to bacteremia. Resistant to linezolid and vancomycin.
<i>E. faecium</i> , Patient #2-1	NR-31909	Isolated from the stool of a human patient prior to bacteremia. Resistant to vancomycin.
<i>E. faecium</i> , Patient #3-1	NR-31912	Isolated from the stool of a human patient having dominance of vancomycin-resistant <i>Enterococcus</i> in the stool but no bacteremia.
<i>E. faecium</i> , E0164	NR-31915	Isolated in 1996 from turkey feces in the Netherlands. Resistant to gentamicin and vancomycin.
<i>E. faecium</i> , E0269	NR-31916	Isolated in 1996 from turkey feces in the Netherlands. Resistant to gentamicin and vancomycin.
<i>E. faecium</i> , HF50106	NR-32054	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin, tetracycline, and vancomycin.
<i>E. faecium</i> , UAA714	NR-32065	Isolated in 1994 in Aix-en-Provence, France. Resistant to vancomycin.
<i>E. faecium</i> , UAA945	NR-32094	Isolated in 1996 in New York, USA. Resistant to vancomycin.
<i>E. faecium</i> , 503	HM-952	Human isolate from the United States. Resistant to vancomycin.
<i>E. faecium</i> , E417	HM-965	Isolated from human blood in Ecuador (2006). Resistant to ampicillin, vancomycin, and displays high-level resistance to gentamicin and streptomycin.
<i>E. faecium</i> , ERV165	HM-970	Isolated from human feces collected in Colombia (2008). Resistant to vancomycin.
<i>E. faecium</i> , E1578	NR-31933	Isolated in 2001 from the feces of a miniature pig in Germany. Resistant to vancomycin.
<i>E. faecalis</i> , SF24413	NR-31971	Urine sample obtained in Michigan, USA. Resistant to erythromycin, gentamicin, and vancomycin.
<i>E. faecalis</i> , SF28073	NR-31972	Isolated in 2003 from a human urine sample obtained in Michigan, USA. Resistant to erythromycin, gentamicin, and vancomycin.
<i>E. faecalis</i> , TX0104	HM-201	Isolated in January 2002 from the blood of a patient with endocarditis at Stamford Hospital in Connecticut, USA. Resistant to vancomycin.
<i>E. faecalis</i> , S613	HM-334	Isolated in 2004 from the blood of a 64 year-old female hemodialysis patient with fatal bacteremia. Resistant to vancomycin.
<i>E. faecalis</i> , R712	HM-335	Isolated in 2004 from the blood of a 64-year-old female hemodialysis patient with fatal bacteremia. Resistant to daptomycin and vancomycin.
<i>E. faecalis</i> , ERV103	HM-934	Isolated from human secretion in Bogota, Colombia (2006). Resistant to vancomycin.

Table 2

The minimum inhibitory concentration (MIC in µg/mL) of salicylanilide drugs (niclosamide, closantel, rafoxanide, and oxyclozanide) and control antibiotics against clinical isolates of vancomycin-resistant *E. faecium* and *E. faecalis*.

VRE strains	Niclosamide	Closantel	Rafoxanide	Oxyclozanide	Linezolid	Ramoplanin	Vancomycin
<i>E. faecium</i> NR-31914	8	8	32	32	1	2	>128
<i>E. faecium</i> HM-968	2	2	8	16	1	1	>128
<i>E. faecium</i> NR-28978	1	2	8	8	1	2	>128
<i>E. faecium</i> NR-31903	4	4	16	4	16	2	>128
<i>E. faecium</i> NR-31909	4	4	16	8	1	4	>128
<i>E. faecium</i> NR-31912	2	4	16	4	1	2	>128
<i>E. faecium</i> NR-31915	1	2	4	2	1	1	>128
<i>E. faecium</i> NR-31916	1	4	8	2	1	1	128
<i>E. faecium</i> NR-32054	2	4	16	4	1	0.25	128
<i>E. faecium</i> NR-32065	1	4	8	4	1	2	>128
<i>E. faecium</i> NR-32094	1	2	8	2	0.5	2	>128
<i>E. faecium</i> HM-952	2	4	16	4	1	2	>128
<i>E. faecium</i> HM-965	4	2	8	16	1	1	>128
<i>E. faecium</i> HM-970	1	2	16	2	1	0.5	>128
<i>E. faecium</i> NR-31933	4	4	16	32	2	2	4
<i>E. faecalis</i> NR-31971	>128	2	16	16	1	2	64
<i>E. faecalis</i> NR-31972	32	1	16	16	1	4	>128
<i>E. faecalis</i> HM-201	32	2	16	16	1	2	>128
<i>E. faecalis</i> HM-334	128	2	16	32	1	2	>128
<i>E. faecalis</i> HM-335	128	2	32	32	1	2	>128
<i>E. faecalis</i> HM-934	128	2	16	32	1	4	>128