THE USE OF ORAL PROPHYLACTICS FOR THE MAINTENANCE OF GUT HEALTH IN TRAVELLERS AND THE EFFECT ON DEVELOPMENT OF ANTIMICROBIAL RESISTANCE.

by

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ABSTRACT

BACKGROUND

Travellers' Diarrhoea (TD) is a significant issue for deployed troops. Antibiotic prophylaxis can be effective, but raises antimicrobial resistance (AMR) concerns. We aimed to evaluate the risk of extended-spectrum ß -lactamase (ESBL) acquisition in the PREVENT TD trial, a double-blind, randomised, placebo-controlled trial comparing once- and twice-daily rifaximin 550mg against placebo (ClinicalTrials.gov: NCT02498301). Volunteers were from UK Armed Forces personnel in British Army Training Unit Kenya (BATUK)

This dissertation presents results on the acquisition of ESBL-producing Enterobacteriaceae in PREVENT TD participants. It also uses bioinformatic techniques and command line interfaces to analyse the presence of antimicrobial resistance genes, virulence factors and construct phylogenetic trees.
It also attempts to identify transmission of strains between individuals.

It then moves on to a further study of TD prevention using an over-the-counter product, Travelan, an anti-*E. coli* immunoglobulin and the conduct of an ongoing randomised control trial including the regulatory approval processes.

MATERIALS

Enrolled UK subjects deployed to BATUK for 6-week periods. 121 participants provided stool samples upon arrival and prior to departure.
Approximately 100mg of stool was incubated in Luria Broth with cefotaxime (10µg/ml) at 37°C for 18 hours. These cultures were then spread onto ESBL Chromoselect agar (Merck) and incubated for 24 hours at 40°C. The presence of growth, was taken as positive for ESBL bacteria. The two rifaximin intervention arms were compared to placebo using Fisher's exact test.

A selection of resistant *E. coli* strains that grew on ESBL Chromoselect agar and sensitive strains that grew on UTI Chromoselect agar were sent for short read sequencing and bioinformatic analysis performed.

RESULTS

A total of 84 of 121 (69.4%) subjects had no ESBL present on arrival in BATUK and were eligible for an assessment of ESBL acquisition. The highest incidence of ESBL acquisition was observed in subjects randomized to the placebo arm; however, the differences were not statistically significant.

	Acquired ESBL	p-value
Placebo	9/24 (37.5%)	--
Rifaximin 550mg once-daily	7/30 (23.3%)	0.37
Rifaximin 550mg twice-daily	6/30 (20.0%)	0.22
Total	22/84 (26.5%)	--

Analysis of those individuals whose first study visit in BATUK found that 30.6% had *E. coli* that grew on ESBL Chromoselect agar. This level is around twice what is seen in previous UK studies.
Rifaximin resistance levels increased from 16.4% to 35.5% during the study. Azithromycin resistance increased from 11.5% to 36.4%

Multi Locus Sequence Typing identified that Sequence Type (ST) 69 was the most common ST and the most frequent *E. coli* phylotype was A. There were Intestinal Pathogenic E. coli associated virulence factors identified in 49/51 (96.1%) genomes. This does not necessarily mean the strains are pathogenic, as many VF work in concert with others to cause clinical disease,

The most common Extended-Spectrum-Beta-Lactamase gene was blaCTX-M-15. Other strains contained genes such as bla-DHA, blaOXA, blaTEM and blaEC, which do not always confer ESBL resistance. The reasons for this are discussed.

Numerous strains had similarities in their plasmids, virulence factors and AMR genes, but were not sufficiently close in SNP distances to say they were the same strains.

CONCLUSIONS

This is the first study to assess risk of ESBL acquisition in travellers after rifaximin prophylaxis. While ESBL acquisition rate was relatively high (26.5%), rifaximin was not associated with an increase in ESBL acquisition. This may be a significant advantage as a prophylactic agent. Additional trials are needed to corroborate these findings.

A follow on randomised controlled trial comparing Travelan and placebo in the maintenance of gut health is underway.

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ABBREVIATIONS

ADMM	Academic Department of Military Medicine
AEIC	Adherent-Invasive *Escherichia coli*
AMR	Antimicrobial Resistance
ASAC	Army Scientific Advisory Committee
BATUK	British Army Training Unit Kenya
B-GOS	β-Galacto-Oligosaccharide
CFU	Colony Forming Units
CMV	Cytomegalovirus
COMBAT	Carriage of Multiresistant Bacteria After Travel
CPE	Carbapenemase producing *Enterobacteriaceae*
CRC	Clinical Research Co-ordinator
CRE	Carbapenem-resistant *Enterobacteriaceae*
CTIMP	Clinical Trial of Investigational Medicinal Product
DAEC	Diffusely adherent *Escherichia coli*
DALYs	Disability Adjusted Life Years
DCC	Data Co-ordination Centre
DEC	Diarrhoeagenic *E. coli*
DMS	Defence Medical Services
DNA	Deoxyribonucleic Acid
DNBI	Disease, Non-Battle Injury
DPA	Data Protection Act 2018
COVID-19	SARS CoV-2 infection
EAEC	Enteroaggregative *Escherichia coli*
EDC	Electronic Data Capture
EIEC	Enteroinvasive *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended Spectrum β-Lactamase
ESBL-E	Extended Spectrum β-Lactamase *Enterobacteriaceae*
ESBL-EC	Extended Spectrum β-Lactamase *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EUCAST	European Committee Antimicrobial Susceptibility and Testing
ExPEC)	Extra-intestinal pathogenic *E. coli*
FGD	Functional Gut Disorder
GDPR	General Data Protection Regulation
GH	Gut Health
HBC	Hyperimmune Bovine Colostrum
HBS	Human Biological Specimens
HE	Hepatic Encephalopathy
HIV	Human Immunodeficiency Virus
HPC	Healthcare Professionals
HUS	Haemolytic Uraemic Syndrome
IBS	Irritable Bowel Syndrome
ICEs	Integrative and conjugative elements
IDCRP	Infectious Disease Clinical Research Program
IND	Investigating New Drug
IMEs	Integrative mobilisable elements

IPEC	Intestinal Pathogenic *Escherichia coli*
IRB	Institutional Review Board
ITT	Intention To Treat
LB	Luria Broth
LB-C	Luria Broth with Cefotaxime
LMIC	Low and Middle Income Countries
LSTM	Liverpool School of Tropical Medicine
MDR	Multi-Drug Resistance
MHRA	Medicines and Healthcare products Regulatory
MICs	Minimum Inhibitory Concentrations
MLST	Multi Locus Sequence Typing
MOD	Ministry of Defence
MODREC	Ministry of Defence Research and Ethics Committee
mRNA	Messenger Ribonucleic Acid
NCBI	National Centre for BioInformatics
NMCP	Naval Medical Center Portsmouth, USA
PaβN	Phe-Arg-β-Naphthylamide
PCR	Polymerase Chain Reaction
RCI	Research and Clinical Innovation
PI	Principal Investigator
PIS	Participant Information Sheet
ReA	Reactive Arthritis
REC	Resistant *Escherichia coli*
REDCap	Research Electronic Data Capture
SCFAs	Short Chain Fatty Acids
SEC	Sensitive *Escherichia coli*
SPL	Specimen Processing Laboratory
SRB	Scientific Review Board
SSI	Surgical Site Infection
ST	Sequence Type
STEC	Shiga Toxin-producing *E. coli*
TD	Traveller’s Diarrhoea
TFUS	Time to First Unformed Stool
UTI	Urinary Tract Infection
UPEC	Uropathogenic *E. coli*
V1	Initial visit for PREVENT TD study
V2	Final visit for PREVENT TD study
VF	Virulence factors
WGS	Whole Genome Sequencing
WHO	World Health Organisation

1. INTRODUCTION

The detrimental effects of infectious diarrhoea on deploying British forces is well documented at least as far back as the Crusades (Bailey 2013).
Diarrhoeal disease remains a significant cause of Disease, Non-Battle Injury (DNBI) for deployed troops. Self-reported Travellers' Diarrhoea (TD) attack rates were approximately 40% (Connor *et al.* 2013) during 2 separate Op HERRICK (British Military operational deployments to Afghanistan 2002 – 2014) tours. The average "days off duty" with TD was 2.8, with an average of 4 days underperforming per episode. This resulted in an (extrapolated) aggregated 68,918 man-days lost to underperformance over 2 x 6 month deployments to Afghanistan.
US soldiers during the first Gulf War, Irag 1991, experienced 97% diarrhoeal disease rate (Kilpatrick 1993). At any one time, 20% of deployed troops would be operationally ineffective (Hyams et al. 1991). Even during peacetime deployments, there are high rates of TD, such as 36% during 2 studies studying prophylaxis in Belize (Salam *et al*. 1994) (Katelaris et al. 1995).
TD continues to be an important area of Military medical importance (Connor and Farthing 1999).

There has been a British Military base in Nanyuki, Kenya since 1945 (https://www.army.mod.uk/deployments/africa). It is known as British Army Training Unit Kenya (BATUK) and hosts deploying troops on exercises of varying duration throughout the year. Historically there has been a high risk of TD, with attack rates of 60% (Connor and Farthing 1999). Over time there has been improvements in the sanitation and facilities in Nanyuki, with a subsequent reduction in TD rates to 21.9% in 2014 (Burns *et al*. 2020). The majority (54.6%) of TD cases reported multiple episodes and 24.9% of cases required medical attention. The strongest risk factor TD was having a colleague with diarrhoea (OR 51.8). This emphasises the likelihood of TD being transmitted between individuals and the impact it may have on individual performance and unit operational capability, if this spread is not controlled with isolation and hygiene precautions.

For commercial travellers, diarrhoeal illness is the most common health impairment (Rendi-Wagner and Kollaritsch 2002). It affects 20-50% of the more than 50 million visitors to developing countries. TD is defined in the literature as the passage of three or more unformed stools in 24 hours with one or more associated symptoms such as; nausea, vomiting, fever, abdominal pain, tenesmus or bloody stool (von Sonnenburg *et al*. 2000). This usually occurs within the first 7-10 days of travel. TD is a self-limiting and mostly benign disease; however, it is uncomfortable and inconvenient for the individuals and disruptive for their group's schedule, with 12-46% of short-term travellers having to change their itinerary (Steffen et al. 2015). This has led to the search for a preventive method.

Guidelines are available from a number of learned societies for the treatment of diarrhoea. The TrEAT TD study (Riddle *et al*. 2017) was a randomised-controlled trial comparing 3 different single-dose antibiotics, combined with Loperamide, for the treatment of travellers' diarrhoea. 339 US and UK service personnel were recruited

from Afghanistan, Kenya, Djibouti and Honduras. Clinical cure was achieved within 24 hours for greater than 70% of cases and non-inferiority was shown between treatment arms.

1.1. TD AETIOLOGY.

TD is usually caused by ingestion of contaminated food and beverages. A number of infectious agents have been identified, with interesting seasonal and geographical differences (Mattila *et al*. 1992).

1.1.1. BACTERIAL PATHOGENS CAUSING TD

As TD more often occurs in resource-limited settings at a distance from traditional laboratory facilities, it is difficult to collect and analyse stool samples and there is thus a knowledge gap on its aetiology. The causal pathogen is identified in only 40-60% travellers' diarrhoea cases (Al-Abri et al. 2005).
A notable study [add reference] involved 579 Finnish adults travelled to Morocco on 2 package tours, one in summer and the other in winter. The researchers set up a laboratory for enteric pathogens in Agadir. 62% of the 60 winter cases and 58% of 11 summer cases identified one or more pathogen. *Campylobacter* was the leading cause of TD (28%) in the winter, but only 7% of the summer. Conversely, Enterotoxigenic *Escherichia coli* (ETEC) was the most common cause (32%) in the summer, but only 8% in winter.

ETEC is one of the leading pathogens associated with TD. In the TrEAT TD study (Riddle *et al*. 2017) ETEC was detected in 40% of diarrhoeal stool samples that were positive for a pathogen by Polymerase Chain Reaction (PCR) (Lalani *et al*. 2018). Globally, it is one of the leading causes of diarrhoea disease, accounting for >10% of all deaths in children less than 5 years old (Kotloff *et al*. 2013).

There are a various different pathogenic *E. coli* and disease is caused in a number of different ways. *E. coli* causing intestinal disease, are broadly known collectively as Intestinal Pathogenic *E. coli* (IPEC); these include:

- Enteroinvasive *E. coli* (EIEC)
- Enteropathogenic *E. coli* (EPEC)
- Enterotoxigenic *E. coli* (ETEC)
- Enterohaemorrhagic *E. coli* (EHEC)
- Enteroaggregative *E. coli* (EAEC)
- Diffusely adherent *E. coli* (DAEC)
- Adherent-Invasive *E. coli* (AIEC) (Kaper et al. 2004)

Other bacterial pathogens are also important causes of diarrhoeal illness and these will be discussed below.

Campylobacter is estimated to be responsible for 7.5 million disability adjusted life years (DALYs) globally (Poly *et al*. 2019) which is surpassed only by rotavirus in diarrhoeal disease. Campylobacteriosis is the most commonly reported bacterial gastrointestinal infection in Europe (Krutova *et al*. 2019). The majority of cases were reported in the Czech Republic, Germany, Spain and the UK (European Food Safety Authority, 2018).

Campylobacter is also associated with several sequelae, including reactive arthritis (Pope *et al*. 2007), Guillain-Barré syndrome (Nyati and Nyati 2013) and chronic gastrointestinal conditions (Riddle *et al*. 2012).

Typhoid fever is a multi-system inflammatory clinical syndrome caused by *Salmonella enterica* subsp. *enterica* serovar Typhi (Wain et al. 2015). It is transmitted via faecal-oral spread, with no known animal hosts (Eng *et al*. 2015). A common presenting complaint is diarrhoea and abdominal pain.

Vibrio cholerae is a Gram-negative intestinal pathogen first isolated by Robert Koch in 1884 (Harris *et al*. 2012). It is a common cause of moderate-severe diarrhoea in endemic areas (O'Ryan *et al*. 2015). It can lead to death by dehydration and electrolyte imbalance (Harris *et al*. 2012). WHO reported 227,3931 cases and 5,654 deaths in 2017 (WHO Weekly Epidemiological Record, 21 September 2018, vol. 93, 38 (pp. 489–500). Yemen accounted for 84% of cases, indicating the rapid spread during outbreaks.

Collection of fresh stool, and subsequent analysis for diarrhoeal pathogens, in remote locations has obvious difficulties, as does their freezing and storage for later analysis. Recently the development of Whatman FTA Elute cards (FTA card, GE Healthcare Life Sciences, Marlborough, MA, USA) have enabled long term storage of stool samples at room temperature (Lalani *et al*. 2018). These cards lyse cells, bind PCR inhibitors and store nucleic acid. Thus, allowing collection and transportation of stool samples from point of illness to testing sites.
As an alternative approach, OMNIgene Gut collection kits (DNA Genotek, Ontario, Canada) allow collection at room temperature for transport to a laboratory up to 7 – 60 days later, depending on the material being studied and thus its stability in a transport medium.

1.1.2. VIRUSES

Viruses account for a minority of TD (Al-Abri et al. 2005). Small round structured viruses, such as norovirus and rotavirus, account for up to 10% of TD cases (Bolivar et al. 1978). They are particularly associated with outbreaks amongst enclosed communities, such as cruise ships and hospital wards (Carling et al. 2009). Viruses such as Cytomegalovirus (CMV) and Human Immunodeficiency Virus (HIV) cause a chronic diarrhoea (Arasaradnam et al. 2018) but fall outside the scope of this thesis as they are not self-limiting conditions.

On the other hand, the presence of viruses within a host gut can be beneficial (Vitetta et al. 2018). Bacteriophages are universally abundant viruses which target specific bacteria without harming other organisms (Wittebole et al. 2014).
The majority of viruses in the gut are bacteriophages (Duerkop and Hooper 2013) such as *Caudovirales* and *Microviridae*. Bacteriophages were posited as a potential treatment for TD as far back as the 1970s and 1980s (Marcuk et al. 1971; Smith and Huggins 1983; Edelman 1985). Their use is widespread in Eastern Europe (Hill et al. 2018). Therapy is mainly directed against chronic bacterial infection such as skin ulcers and respiratory infections. There are currently no successful treatment studies using bacteriophages against TD.

1.2. PREVENTIVE INTERVENTIONS

The development of effective approaches to maintain gut health and prevent TD is a long-standing requirement for both civilian and military travellers. Current strategies include the provision of education prior to travel. This focuses on reducing risky behaviours (such as avoiding tap water, undercooked meat, unwashed fruits and vegetables.) However, controlling exposure to indigenous food and water is a challenge on short and small-scale overseas travel and has failed to show benefit in multiple studies (Shlim 2005). There is unfortunately little evidence in the literature to support the oft-quoted adage of “boil it, cook it, peel it, or forget it.”

1.2.1. VACCINES

Vaccines have been proposed as a preventive intervention for TD (Adeleke et al. 2021). A vaccine is “any preparation of immunogenic material suitable for the stimulation of active immunity in animals without inducing disease. Vaccines may be based on dead or attenuated microorganisms; altered toxins (toxoids); or viruses” (Cammack *et al*. 2008). Modified mRNA is a recently developed method of vaccine creation as shown in recent years by the production and global utilisation of a SARS-CoV-2 (the causative agent of COVID-19) vaccine (Mulligan et al. 2020). The first vaccine was developed in 1798 by Jenner, who noted the ability of cowpox to prevent smallpox in milk maids (Jenner 1798). Despite many success stories in eradication of communicable disease, most recently the WHO Global Polio Eradication Initiative (Minor 2014), effective TD vaccination has proven more difficult. An ETEC vaccine is in development (Khalil et al. 2023) and may enter Phase 3 trials in the near future.

1.2.2. DAILY PROPHYLACTICS

1.2.2.1 Antibiotics

Bacterial enteric pathogens are the most common cause of TD (Al-Abri et al. 2005). Antimicrobial chemoprophylaxis has proven effective in the prevention and rapid relief of TD. Prophylactic antimicrobials aim to eliminate pathogens prior to their causing symptomatic TD.

Clinical trials of prophylaxis against TD date back to the 1970s, starting with doxycycline 100mg daily (Sack *et al*. 1978; Sack *et al*. 1979). Unfortunately, it was not as effective in an area of high antimicrobial resistance (Sack *et al*. 1984). If the strains in an area are susceptible to the antibiotic, chemoprophylaxis will prevent 80 to 90% of TD (DuPont and Ericsson 1993).

Rifaximin is a semi synthetic, poorly absorbed derivative of rifamycin. It contains an additional pyrido-imidazole ring, thus minimising systemic absorption (Huang and DuPont 2005). It has broad spectrum antibacterial effect against both Gram-positive and Gram-negative bacteria, including aerobic and anaerobic bacteria from the human gut (Scarpignato and Pelosini 2005). It inhibits RNA synthesis, by binding to the β-subunit of DNA dependent mRNA polymerase (Hopkins *et al*. 2014). It is most effective against *E. coli* which is the most commonly isolated cause of TD (Turunen and Kantele 2021).

Rifaximin has been evaluated as TD chemoprophylaxis in several studies. Meta-analyses of available randomised, controlled studies using rifaximin TD chemoprophylaxis (total 604 subjects enrolled) in field settings revealed a pooled

protective efficacy of 67% (95% CI: 55-76%) when compared to placebo (DuPont et al. 2005; Alajbegovic et al. 2012; Ng et al. 2017).
In a treatment study of international travellers to Mexico and Jamaica, the safety profile of rifaximin was equivalent to placebo (DuPont et al. 2001; DuPont 2005). Rifaximin has been shown as effective in human challenge models against shigellosis (Taylor et al. 2006). However, rifaximin was unsuccessful in human challenge models against *Campylobacter* (Rimmer *et al*. 2017). In a 2014 study of *in vitro* activity of rifaximin against *Campylobacter* isolates, 81/90 (90%) were rifaximin resistant at >128mg/L (Hopkins *et al*. 2014).

Rifaximin was selected for use in the PREVENT TD study at a dose of 550mg, which is higher dose than used in previous prophylaxis studies (DuPont et al. 2005). Rifaximin is licensed and regularly prescribed in the UK for hepatic encephalopathy (Butterworth 2019). The treatment intent is reduction in ammonia production by intestinal bacteria (Morgan et al. 2007). The prophylactic dose for hepatic encephalopathy in the UK is 550mg twice daily and has a good safety profile (Maclayton and Eaton-Maxwell 2009).

A perennial concern with widespread regular use of any antimicrobial, is the development of Antimicrobial Resistance (AMR). Rifaximin resistance develops through 3 different mechanisms:

1. Mutations in highly conserved regions of the *rpoB* gene which encodes the β-subunit of mRNA polymerase (Tupin *et al*. 2010).
2. Acquisition of plasmid-related *arr* genes (Baysarowich *et al*. 2008). These genes encode ADP-ribosyltransferases, which inactivate rifamycins.
3. Overexpression of Phe-Arg-β-Naphthylamide (PAβN) inhibitable efflux pump, which reduces cellular accumulation, in leading to resistance (Pons *et al*. 2012).

Resistance mutations are mostly single amino acid substitution (Kothary *et al*. 2013). In an experiment involving repeated passages in a culture of rifaximin-resistant *E. coli* 10/12 (83%) strains remained resistant (Kothary *et al*. 2013). The loss of resistance was due to a point mutation and reversion to wild type. This indicates the resistance mutation appears stable in the absence of antibiotics.

Antibiotic therapy is able to produce profound alterations to the gut microbiome composition (Ponziani *et al*. 2017). Antibiotic treatment also reduces taxonomic richness and diversity, which does not always exactly return to the baseline upon cessation of antibiotic therapy, but more often a slightly different state (Dethlefsen and Relman 2011).
Rifaximin can also downregulate the inflammatory response triggered by gut microbes (Darkoh *et al*. 2010) and alter bacterial virulence by inhibition of adhesion, internalisation and translocation (Jiang et al. 2010). In a mouse model, the abundance of beneficial bacteria, such as lactobacilli (Xu *et al*. 2014) was increased by administration of rifaximin..

1.2.3. Microbiome modulation

Modulation of the gut microbiome is a potential method of reducing TD risk. This is likely due to a combination of mechanisms (Vogt and Finlay 2017). The species that make up the gut microbiota can include bacteria that may compete with invasive

pathogens for nutrients (Johansson et al. 2013). The gut microbiota may also directly inhibit virulence of diarrhoeal pathogens (Antunes et al. 2014).
Underlying gut microbiome variations between different groups are thought to account for the differences in TD rates between groups who have grown up in contrasting environments, such as Gurkhas (Nepalese who join the British Army) and UK born non-Gurkha soldiers, who have very differing rates of TD in unpublished but internally reported studies. British born soldiers had diarrhoeal attack rates of 40-60%, whilst Nepalese born Gurkhas had TD rates of 11% (Connor and Gutierrez 2013).
Arumugam and colleagues recognised differences in gut microbiome, when they defined enterotypes (Arumugam et al. 2011). In their observational study, gut microbial composition varied between different nationalities, which is likely related to dietary habits, lifestyle, genetic components and environmental factors (Mobeen et al. 2018).
Ethnic origin of individuals may be an important factor in microbial diversity (Deschasaux et al. 2018). In a 2018 paper, Deschasaux and colleagues identify that Dutch nationals have the largest α-diversity of their gut microbiome and individuals from South-East Asia have the smallest.
In germ-free mouse models, the gut microbiota demonstrates a protective effect on diarrhoea (Kamada et al. 2012). Differences in the microbiome also has an effect on the likelihood of acquiring multidrug-resistant Enterobacteriaceae (Leo et al. 2019).

1.2.3.1. Probiotics

Probiotics are dietary supplements containing live or attenuated yeast or bacteria, such as *Saccharomyces boulardii* and *Lactobacillus spp.*) They exert protective effects by enhancing the integrity of the mucosal barrier, modulating the immune system and limiting the growth of pathogenic microbes. They metabolise complex carbohydrates, thus producing lactic acid and Short Chain Fatty Acids (SCFAs), that reduce bacterial translocation and maintaining the integrity of the intestinal epithelium by stimulating the synthesis of mucin (Rolfe 2000; Burger-van Paassen et al. 2009; Lewis et al. 2010).
In a meta-analysis by McFarland (McFarland 2007) 12 randomised efficacy trials using *Saccharomyces* and *Lactobacillus* were assessed for GH disruption prevention. Overall, probiotic use was associated with a modest decrease in TD risk (RR: 0.85 [95% CI: 0.79-0.91]; p<0.001.). An updated adaptive meta-analysis in 2017 including 11 RCTs again showed a modest reduction in TD using probiotic prevention (RR: 0.86 [95% CI, 0-80-0.92]) (Bae 2018).
A Cochrane review of probiotics in the treatment of acute infectious diarrhoea did not show any positive effect in the treatment of TD (Collinson et al. 2020).

1.2.3.2. Prebiotics

Prebiotics are oligosaccharides that remain undigested in the human gut until they are fermented by colonic bacteria. SCFAs produced during fermentation benefit the host by modulating cytokine production and stimulating growth and/ or activity of bacteria that have been proposed to contribute positively to gut health, such as lactobacilli and *Bifidobacterium* (Bouhnik *et al*. 1997).

Two randomised trials of prebiotics have evaluated efficacy for diarrhoea prevention. In 244 UK travellers to intermediate/ high risk TD regions, fructooligosaccharides (FOS) failed to reach preventive efficacy endpoint of 20% (46% placebo vs. 38%

FOS; $p>0.1$). It was also associated with increased side effects, such as flatulence and increase stool frequency (Cummings et al. 2001). However, it did improve the study participants sense of "well-being." A criticism of the study was the absence of a standard definition of TD as an outcome measure.
A further study of β-Galacto-Oligosaccharide (B-GOS) showed a protective efficacy against TD. In a double-blinded, placebo controlled, randomized parallel group design, 159 healthy volunteers traveling to areas associated with medium or high risk for TD between 10 and 60 days were divided in two groups consuming either B-GOS or placebo. The test article was consumed for a period of seven days prior to travel and daily during travel. B-GOS reduced TD incidence (defined as ≥ 3 loose stools per day) compared with placebo [19/81(23.4%) vs. 30/78 (38.5%), a relative risk reduction (RRR) of 39%; absolute risk reduction (ARR) = 15%]. Duration of TD was also reduced in the treatment group (2.4 days compared to 4.6 days in the placebo group ($p<0.05$)) (Drakoularakou et al. 2010).

1.2.3.3. Passive immunoprophylaxis.

Hyperimmune Bovine Colostrum (HBC) is produced by repeated immunisation of pregnant cows to produce microbe-specific IgG. This pathogen-specific IgG is thought to bind pathogenic antigens in the intestinal lumen, thus inhibiting the binding of toxins and enteropathogens to intestinal receptors. This limits disruption to the microbiome and maintains gut health (Hurley and Theil 2011).
Human challenge studies have found HBC effective in preventing diarrhoea caused by cryptosporidiosis, *Shigella flexneri* and ETEC infection (Okhuysen et al. 1998; Tacket et al. 1992; Savarino et al. 2017). HBC are specific to each pathogen and its effective use thus relies on understanding of the epidemiology of diarrhoeal pathogens in specific regions in which it can be employed.

1.3. ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) follows antimicrobial discovery due to the evolutionary drive and ability by bacteria to survive (Meletis 2016). Susceptible bacteria are able to acquire genes horizontally and implement the necessary mechanisms for their survival. As explained in the introduction, this is an increasing problem worldwide. Multidrug resistance (MDR) is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al*. 2012).

Antibiotic misuse is a leading cause of AMR worldwide (Health and Services 2013). In the UK, a National Action Plan was published in 2019 (Courtenay *et al*. 2019). It sets the goal of reducing UK antimicrobial use in humans by 15% by 2024, which includes a 25% reduction in antibiotic use in the community from a 2013 baseline.

The most commonly prescribed antibiotics in an outpatient setting in the USA between 2013 and 2015 were azithromycin, amoxicillin, amoxicillin/ clavulanate, ciprofloxacin and cephalexin (Durkin *et al*. 2018). Seasonal variations are often seen in antibiotic prescriptions (Patrick *et al*. 2004) but there was no change in annual total antibiotic prescriptions. In the UK, similar rates of antibiotic prescribing were seen and a large proportion were found to be inappropriate, with up to 67.3% of antibiotic prescriptions for otitis externa and 38.7% of antibiotics for upper respiratory tract infection deemed inappropriate (Nowakowska *et al*. 2019). This was either due to viral infection or the use of the wrong antibiotic based on empirical guidelines.

β-lactams are the most used antibiotics worldwide (Meletis 2016). They include penicillins, cephalosporins, monobactams and carbapenems, in escalating potency. Resistance for each of these classes depends on the presence of AMR genes. Since the discovery of Penicillin by Sir Alexander Fleming (Fleming 1929) β-lactam use has led to development of resistance, mainly due to the secretion of β-lactamases (Rapp and Urban 2012).
Tazobactam and clavulanate can be combined with penicillins, to inhibit TEM, CTX-M & SHV ESBLs (Bush *et al*. 1993) (Drawz and Bonomo 2010).

β-lactamases are enzymes that break down β-lactam antibiotics making them ineffective. They are categorised into 4 classes based on their molecular structure, known as Ambler classification (Ambler 1980). These are classes A-D and encoded by "*bla*-" genes. Particularly problematic among the β-lactamases are the Extended Spectrum β-lactamases (ESBLs), which confer resistance to penicillins and cephalosporins. The main ESBL genes are *blaCTX-M*, *blaTEM* and *blaDHV* groups (Yu *et al*. 2007). Finally, carbapenem-resistant Enterobacteriaceae (CRE) produce carbapenemases that confer resistance to practically all β-lactam antibiotics, including the carbapenems.
Resistance to β-lactams is reported worldwide (D'Andrea *et al*. 2013).

AMR genes also exist in non-exposed bacteria and their existence predates the use of antibiotics by humans (Van Goethem et al. 2018) due to the presence of natural antibiotics. However, extensive and widespread AMR did not develop as the naturally occurring concentrations of antimicrobials was an order of magnitude lower than that used in clinical practice, reducing selective pressures (Spagnolo et al. 2021).

The growth in ESBL prevalence has been mainly due to the increasing use and success of cephalosporins to treat Gram-negative pathogens since the 1980s (Baron *et al*. 2014). Resistance through the production of β-lactamase is mainly achieved by Gram-negative bacteria (such as *E. coli*), which can produce a variety of hydrolytically active β-lactamase, which vary in their range of activity and enzymatic profile (Du *et al*. 2002). MDR *E. coli* and *Klebsiella pneumoniae* clinical isolates have risen during the 21st century (Dunn et al. 2019). The main resistance mechanism is drug inactivation, which is achieved by hydrolysis of the β-lactam ring on the antibiotic molecule by the ESBL enzyme (Musicha et al. 2017). ST131 is a common and emerging ESBL *E. coli* lineage. There is evidence that *E. coli* ST131 acquires and maintains carbapenemase resistance genes and plasmids (Peirano *et al*. 2014).

Once colonised with an ESBL-producing *E. coli*, these strains can remain persistent despite decolonisation regimes (Bar-Yoseph *et al*. 2016). A systematic review and meta-analysis comparing decolonisation strategies across international healthcare settings showed rates of ESBL colonisation at 35.2% at 12 month follow up without decolonisation, and 37.1% with decolonisation. Interestingly, a strategy described by Rieg and colleagues (Rieg *et al*. 2015) was rifaximin 400mg twice daily for 2-3 weeks. Rifaximin decolonisation treatment led to Extended Spectrum β-Lactamase *Enterobacteriaceae* (ESBL-E) eradication in 9/15 (60%) of patients.

AMR development complicates the treatment of TD. In *vitro* selection and quantification of *E. coli* rifaximin resistance rates demonstrates 26 of 28 diarrhoeagenic *E. coli* developed resistance at an MIC > 256mg/L (Ruiz *et al*. 2008). The frequency of rifaximin-resistant selection mutants ranged from 5.7 x 10^{7} to 1.6 x 10^{6} for Enterotoxinogenic *E. coli* (ETEC) and 2.0 x 10^{8} to 9.3 x 10^{8} in the case of Enteroaggregative *E. coli* (EAEC). This did not affect the *E. coli* MICs for chloramphenicol, nalidixic acid or ciprofloxacin.
The macrolide azithromycin is the primary antibiotic to treat TD in deployed soldiers. It is a broad-spectrum macrolide antibiotic (Parnham *et al*. 2014).
Azithromycin was developed in the 1980s as a semi-synthetic derivative of erythromycin (Bright and Hauske 1984). It inhibits bacterial protein synthesis by interrupting assembly of 50S ribosomal sub-unit. This gives azithromycin an extended range of antibacterial actions (Hoffmann *et al*. 2007). It also has a good safety profile, leading to its widespread use (Milberg *et al*. 2002).
It was shown as effective as single dose treatment for TD in the previously mentioned TrEAT TD study (Riddle *et al*. 2017) and is the first line antibiotic for treatment of TD in a deployed military population.

Azithromycin resistance is a growing problem. The half-life of Azithromycin is 68 hours, which leads to prolonged exposure of the antibiotic and thus increases the chance of resistance developing (Derbie et al. 2020). Genes such as *mph(A) 2* (UniProtKB – A0A1U9X4I1 ECOLX) play a role in development of Azithromycin resistance. It is one of at least 15 gene subtypes of the Macrolide Phosphotransferase family, which have been designated *mph(A)* to *mph(O)* (Golkar et al. 2018). These are encoded on mobile genetic elements and exert their effect by phosphorylating the hydroxyl moiety on decosamine or mycaminose groups, which are present in all macrolides. In a study of 343 *E. coli* isolates the presence of *mph(A)* gene was associated with higher azithromycin MIC levels. 93% isolates with *mph(A)* as a single macrolide resistance gene had MICs >= 32mg/L (Gomes *et al*. 2013).

In recent work, rifaximin resistance was studied in *Enterobacterales* that were also resistant to third-generation cephalosporins (Baumert *et al*. 2020) and identified 9 clinical *E. coli* isolates out of 342 strains. Rifaximin-resistant strains are thus rare among ESBL-positive bacteria, but there is an increased risk that this combined resistance may emerge in those taking daily rifaximin.

ESBL carriage is associated with worse healthcare outcomes, including increased mortality (John et al. 2019). It is also associated with increased healthcare costs, increasing by between 25-48% (Maslikowska et al. 2016) mainly due to prolonged hospital stay and increased infection control measures (Esteve-Palau *et al*. 2015).

ESBL carriage leads to increased risk of surgical site infections (SSI) during elective surgery (Dubinsky-Pertzov et al. 2019). This leads to the possibility that prophylactic antibiotics given for surgery (commonly penicillin) will not be effective.

A study from 2014 estimated the prevalence of CTX-M ESBL-E faecal colonisation in the general adult population (McNulty *et al*. 2018). 2,430 individuals participated, with an estimated prevalence in England of 7.3% (95% CI 5.6-9.4%.) There were differences across the country, with 16% colonisation in Birmingham. Risk factors for

colonisation were being born in the Indian sub-continent or recent travel overseas to regions with high environmental ESBL prevalence.

ESBL-producing *E. coli* (ESBL-EC) are found increasingly frequently, mainly in nosocomial settings in the UK (UK-HSA 2023). However, 19% of healthy volunteers in an isolated setting were found to carry ESBL-EC (Mathai *et al*. 2015). Concerningly, there is increasing extra-intestinal ESBL-EC seen in UK hospitals causing bacteraemia (Alhashash *et al*. 2013).

This compares to 9% ESBL-EC carriage in an observational study of all patients admitted to a London hospital (Otter *et al*. 2019). ESBL-EC was the most common ESBL-E. Whilst one would suspect those who attended hospital to be different demographics and have increased prior exposure to antibiotics compared to the general population, these observational studies of prevalence give an indication of the abundance of ESBL in the population.

1.3.1. ESBL SITUATION IN LOW AND MIDDLE INCOME COUNTRIES (LMICs)

There are higher levels of ESBL-producing Enterobaceriaceae (ESBL-E) carriage in low and middle income countries (LMICs) (Darlow and Hope 2021) and thus overseas travel is a common risk factor for acquisition of ESBL-E (Carattoli 2013). This was confirmed by the Carriage Of Multiresistant Bacteria After Travel (COMBAT) study (Arcilla *et al*. 2017), which recruited 2,001 Dutch travellers and 215 non-travelling household members. Stool samples were collected before and sequentially after foreign travel. 34.3% travellers acquired ESBL-E. Colonisation continued for a median of 30 days upon return, and 11.3% remained colonised at 12 months. In the COMBAT study, travellers to Southern Asia had the greatest risk of ESBL-E acquisition ((75.1%). Those travelling to middle and eastern Africa, including Kenya, had a 27.8% risk.

French Military deploying overseas also saw high rates of ESBL-E acquisition (Maataoui *et al*. 2019). The highest rate was those travelling to Afghanistan (88%) and the lowest rate was those travelling to French Guiana (5%).

A study of 20 Health Care Professionals (HCP) visiting Laos looked at transmission of ESBL *E. coli* between a cohort that was travelling and living together (Kantele *et al*. 2021). Colonisation rates at the end of the study were 70%, but via daily sampling, it was revealed that all participants had acquired ESBL-E at some point during the study. 95% of participants acquired 2 or more MDR strains and some participants acquired 7 strains over the course of the study. The majority of ESBL-E identified were *E. coli* (53/83 = 63.8%). In this relatively small cohort, ESBL-E were found in 7 (35%) of initial participant stool samples. This high baseline carriage rate could be explained by their professional occupation as HCP, who are known to have higher rates of ESBL-E carriage (Prestinaci et al. 2015). A number of the participants had also visited the region within the last year, which could also lead to prolonged ESBL-E carriage

A study of neonates admitted to hospital in Kilifi, Kenya showed an ESBL-E prevalence of 10% on admission (Kagia *et al*. 2019). 55% of those admitted without ESBL-E carriage acquired it during their stay. This could be the result of abundant

ESBL-E within the hospital or the use of broad-spectrum antibiotics and the subsequent evolutionary pressure upon the previously non-resistant *Enterobacterales*. A separate study was published of organisms causing Urinary Tract Infections (UTI), a common clinical source of extra-intestinal ESBL-E infection, from patients at Kenyatta National Hospital, Nairobi, Kenya (Magale *et al*. 2015). The researchers analysed 948 urine samples, of which 226 had significant growth. ESBL isolates were found in at least 30% of the samples.

There have also been studies of food contamination with ESBL-E. A 2010 study of commercially available raw chicken breasts purchased in Utrecht, Netherlands (Leverstein-van Hall *et al*. 2011) found 92/98 (94%) of samples contained at least one ESBL-E *E. coli.* This is a potential vector for ESBL-E entry into the individuals. Despite low levels of human antimicrobial use in the Netherlands (van den Broek d'Obrenan *et al*. 2014), there are higher levels of antibiotic use in poultry in the Netherlands compared to any other European country (Grave et al. 2010). Another study from 2011, in Swiss Pork demonstrated 15.2% ESBL-E carriage rates (Geser *et al*. 2011).
There was also an ESBL-E prevalence 5.2% in raw root vegetables (van Hoek et al. 2015) in the Netherlands. This is another route of possible entry into individuals.
A study into AMR profiles in ready to eat food in Kibera, Kenya (the largest slum in the capital Nairobi) showed prevalence in 38% (Maina *et al*. 2021). This food consisted of cooked vegetables, meat, fish and various maize. *E. coli* was the most common AMR bacteria isolated (26%).

1.4. BIOINFORMATIC TECHNIQUES

Over the last two decades, whole genome sequencing (WGS) has become an important tool study the dissemination of antibiotic-resistant bacteria. WGS is a laboratory process to determine almost all the DNA sequence of an organism and analyse its genetic variation (Ng and Kirkness 2010). It demonstrates analytical superiority to most other forms of DNA-based testing (Austin-Tse et al. 2022).

Clinicians have long been hesitant to implement WGS in outbreaks due to the high cost and cumbersome early technologies (Quainoo et al. 2017), but recent advances in sequencing platforms and analysis tools have sped up this process and have driven down costs.

WGS can be used in to identify virulence and resistance genes (Leopold et al. 2014). Analysis of the genome sequence can be performed using bioinformatic tools to identify AMR genes, plasmids and multi-locus sequence typing to characterise bacterial isolates (Maiden et al. 1998). This can be coupled with phenotypic resistance testing in laboratory conditions to determine resistance patterns.

AIM AND OBJECTIVES

The aim on this thesis is to study the diversity of multidrug-resistant *E. coli* colonising British soldiers in Kenya and to set up a further study to minimise the risk of TD in this group.
The specific objectives of this study are thus:

1. Determine the prevalence of antibiotic-resistant *E. coli* and other Enterobacteriaceae in soldiers taking rifaximin prophylaxis deploying to Kenya.
2. Analyse the diversity of *E. coli* strains using genomic sequencing
3. Preparation of a Clinical Trial for the use of over-the-counter nutraceuticals in the prevention of Traveller's Diarrhoea (TD) and the maintenance of gut health (GH.)

CHAPTER 2. DETERMINATION OF THE PREVALENCE OF ANTIMICROBIAL RESISTANT *E. coli* AND OTHER ENTEROBACTERALES.

PREVENT TD (ClinicalTrials.gov: NCT02498301) was a multi-site, double-blind, placebo-controlled trial of deployed Military personnel, deployed to Kenya, were assigned to either rifaximin 550mg twice daily, or once daily with placebo, or a control placebo group. There was 1:1:1 randomisation and study participants took the study drug for 42 days. Participants kept stool and symptom diaries and were collected for analysis upon study completion. The Primary end point was time to first unformed stool (TFUS)). In UK Military personnel deploying to Kenya, the twice daily regimen vs placebo resulted in a significant decrease in TD episodes (1.6% vs. 11.9%; p=0.025) with no increase in adverse events between groups (Gutierrez et al. 2020). Here we aimed to determine the presence of *E. coli* and other *Enterobacterales* in the stool samples collected in the PREVENT TD study.

2.1. METHODS AND MATERIALS

During the PREVENT TD trial, 121 paired stool samples (totalling 242 samples) were collected and stored in 5ml Eppendorf tubes (Hamburg, Germany) at -80°C, without the addition of preservatives. Study participants provided a sample, within 2 days of arrival in Kenya (V1) and a further sample, prior to leaving Kenya, following 6 weeks of either rifaximin 550mg once daily, twice daily or placebo (V2) These were transferred from Kenya to Birmingham, using Crédo Cubes (Pelican Biothermal, Leighton Buzzard, UK) frozen to -80°C with temperature monitoring throughout. The samples were stored at The BioHub, Birmingham Research Park (Edgbaston, UK) in accordance with the regulations set out by the Human Tissue Act 2004. The analysis was completed at Institute of Microbiology and Infection, University of Birmingham, UK. Following this, the samples were shipped to our collaborators laboratories at Washington University, St. Louis, MO, USA and Naval Medical Research Center (NMRC), Bethesda, MD, USA. There further work including metagenomic shotgun sequencing will be performed, which was outside the scope of this project.
Each subject was given a unique PU number to maintain anonymity, comply with ethical approvals and good clinical practice. Ethical approval was granted by Ministry of Defence Research and Ethics Committee (MODREC) as an amendment to the original PREVENT TD protocol (770MODREC16 v11.0).
The 220 soldiers that were approached to take part in this study were deployed to Kenya in 4 consecutive cohorts:

- PU0001 – PU0033. Aug 2018 – Oct 2018. Cohort 1.
- PU0034 – PU0096. Oct 2018 – Nov 2018. Cohort 2.
- PU0097 – PU0156. Jan 2019 – Feb 2019. Cohort 3.
- PU0157 – PU0220. Feb 2019 – Apr 2019. Cohort 4.

The regiments are based in 4 separate parts of the UK, but the soldiers are not necessarily recruited from that specific region of the country.

For the experiments described here, the stool samples were worked up to isolate *E. coli* and ESBL-producing *E. coli* (Fig. 1).

On Day 1, samples were thawed temporarily on ice, such that a small piece could be removed (range 0.1g±0.01g) before being diluted 10-fold and 100-fold in Phosphate Buffered Solution (PBS). These dilutions were then also plated onto MacConkey agar, then incubated at 37ºC overnight.
100µl of the 10^{1} dilution was added to 3ml of Luria Broth (LB) supplemented with cefotaxime (10µg/ml.) This was incubated overnight at 37ºC to allow enrichment for ESBL-producing strains.
A second 0.2±0.02g sample was refrozen in 15% glycerol for future analysis, if required.

After incubation, the MacConkey agar plates were counted to calculate the total coliform Colony Forming Units (CFUs, Fig 3.)
For MacConkey agar samples that did not demonstrate growth initially, 30µg of the 10^{1} dilution stool sample was added to 3ml LB for overnight enrichment. This was then spread onto MacConkey agar and incubated for a further 24 hours at 37ºC.
Any LB with Cefotaxime (LB-C) enriched samples that demonstrated growth were streaked onto an ESBL Chromoselect (Sigma Aldrich, Gillingham, UK) agar plates and again incubated overnight at 37ºC, after which, ESBL Chromoselect plates were examined with any *E. coli* colony showing as a pink to purple (Fig 1.) The other common Gram-negative growing on ESBL Chromoselect agar is *Klebsiella,* which will appear as a bluish green colony (Glupczynski *et al*. 2007).

Three individual colonies were picked from each plate and grown in LB overnight after which they were stored at -80 in 15% glycerol for cryopreservation.
These samples were then processed by MicrobesNG (https://microbesng.com) for whole genome sequencing and the analysis of these data will be discussed in the next Chapter. The assumption was those colonies that were picked from the ESBL Chromoselect plate are ESBL-producers and are referred to as such later.

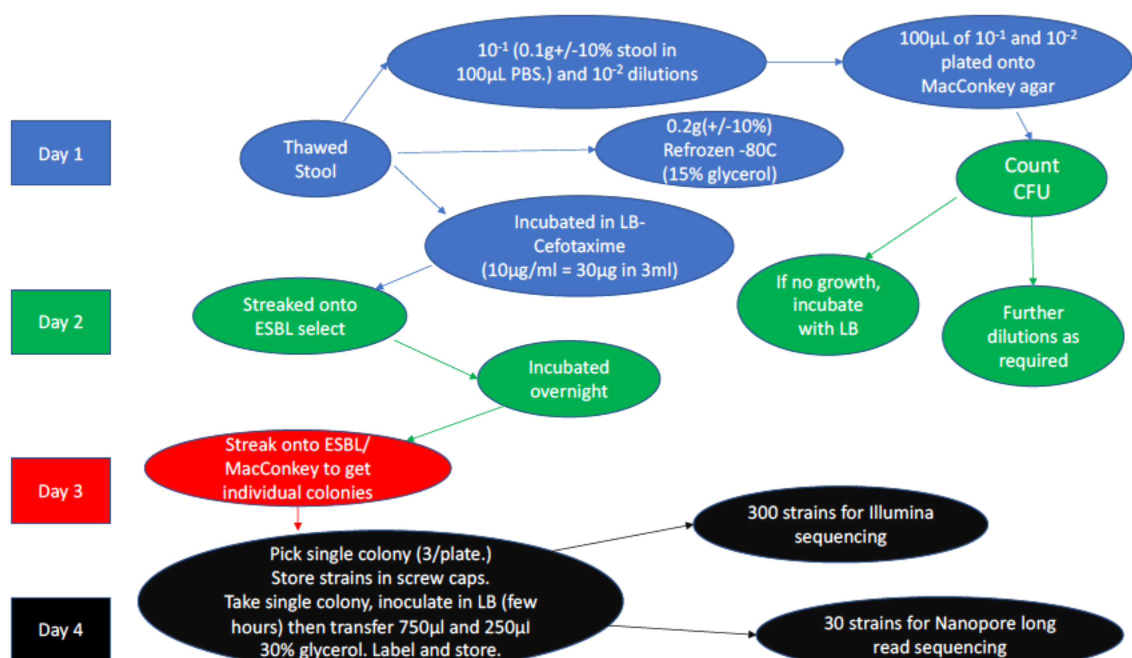


Figure 1. Laboratory processing of PREVENT TD stool samples.

Prior to processing the samples as described above, an *E. coli* reference strain (MG1655) and a ESBL-producing *E. coli* (EC5) were streaked onto MacConkey, UTI Chromoselect and ESBL Chromoselect agars (Fig 2) as controls of colony morphology.

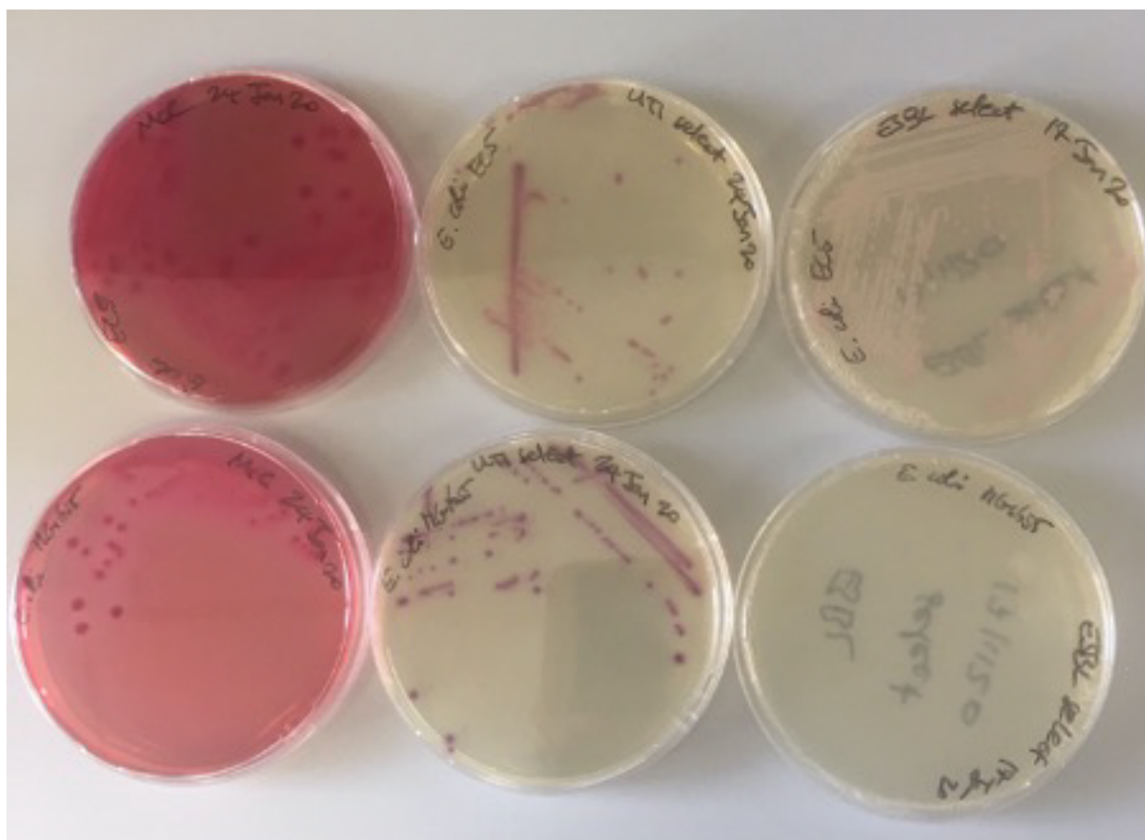


Figure 2. MacConkey, UTI Chromoselect and ESBL Chromoselect agars demonstrating growth of ESBL-producing *E. coli* EC5 (top row) and the reference strain *E. coli* MG1655 (bottom row).

The UTI Chromoselect Agar facilitates identification of different contrasting colonies based on their colours, which are produced by their reaction with chromogenic substrates. The chromogenic substrates are cleaved by enzymes produced by *Enterococcus* species, *Escherichia* and coliforms. β-glucosidase cleaves chromogenic substrate resulting in the formation of blue colonies. Coliforms produce purple-coloured colonies due to the cleavage of β-D-galactosidase both chromogenic substrates. Culture characteristics from UTI Chromoselect agar product literature is presented below (table 1).
(https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/346/211/16636dat.pdf).

Cultural characteristics after 24 hours at 35-37 ºC.

Organisms (ATCC)	Growth	Color of colony	TDA	DMACA
Escherichia coli (25922)	+++	pink-red	-	+
Proteus mirabilis (10975)	+++	light brown	+	-
Klebsiella pneumoniae (13883)	+++	blue to purple (mucoid)	-	-
Pseudomonas aeruginosa (27853)	+++	colorless	-	-
Staphylococcus aureus (25923)	+++	golden yellow	-	-
Enterococcus faecalis (29212)	+++	blue (small)	-	-

Table 1. Phenotypic appearance of coliforms on UTI Chromoselect agar.

2.1.1. ANTIBIOTIC SUSCEPTIBILITY TESTING

Minimum inhibitory concentrations (MICs) against rifaximin and azithromycin (the antibiotic of choice in the Clinical Guidelines for Operations used by deployed UK Military personnel) were determined using the agar dilution method approved by the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews 2001). To perform agar dilution susceptibility testing, we used 96 well plates (Fisher Scientific, Loughborough, UK) and a MAST URIDOT system (Mast Group, Liverpool, UK) to inoculate plates.

We determined MICs for rifaximin and azithromycin using 150 *E. coli* strains in total from 70 individuals. These were divided onto two separate 96 well plates, avoiding the edges of each plate as results are imperfect in the margins of a plate.

The plates were prepared with IsoSensitest agar mixed with antibiotics at increasing concentrations. A control plate without antibiotic was also used to confirm the viability of the strains.

Each strain was freshly streaked on medium and incubated at 37°C overnight, then a single CFU was used to incubate 5ml LB, which was incubated at 37°C overnight, to achieve a cell density of approximately 10^9 CFU/ml. This culture was then diluted 1:100 by adding 100µL to 9,900µL of LB. The URIDOT took 1µL from each cell suspensions. The 96 well plate with multi pin innoculator took 1µL of LB culture grown overnight

The plates were again incubated overnight at 37°C and a record made of the concentration of antibiotics at which *E. coli* growth was inhibited.

2.2. RESULTS

242 stool samples from 121 participants were analysed. 164/242 (67.7%) had growth on MacConkey agar. A further 38/78 (48.7%) had growth on MacConkey agar after plating the enrichment culture in LB. Coliforms were thus cultured from 202/242 (83.4%) of day 2 stool samples.

It was not possible to count CFUs for those samples that required enrichment. There were 94 paired samples from 47 individuals, that did not require enrichment. Comparison of CFUs was made using paired t-test and this did not show a significant difference for any of the study groups (Fig 3.)
The mean CFU count at the end of the study of each group was then also compared via a Kruskal-Wallis test (as the data was assessed to be non-parametric). The data had an H-value of 5.395, but again the difference was found to be non-significant (p = 0.07).

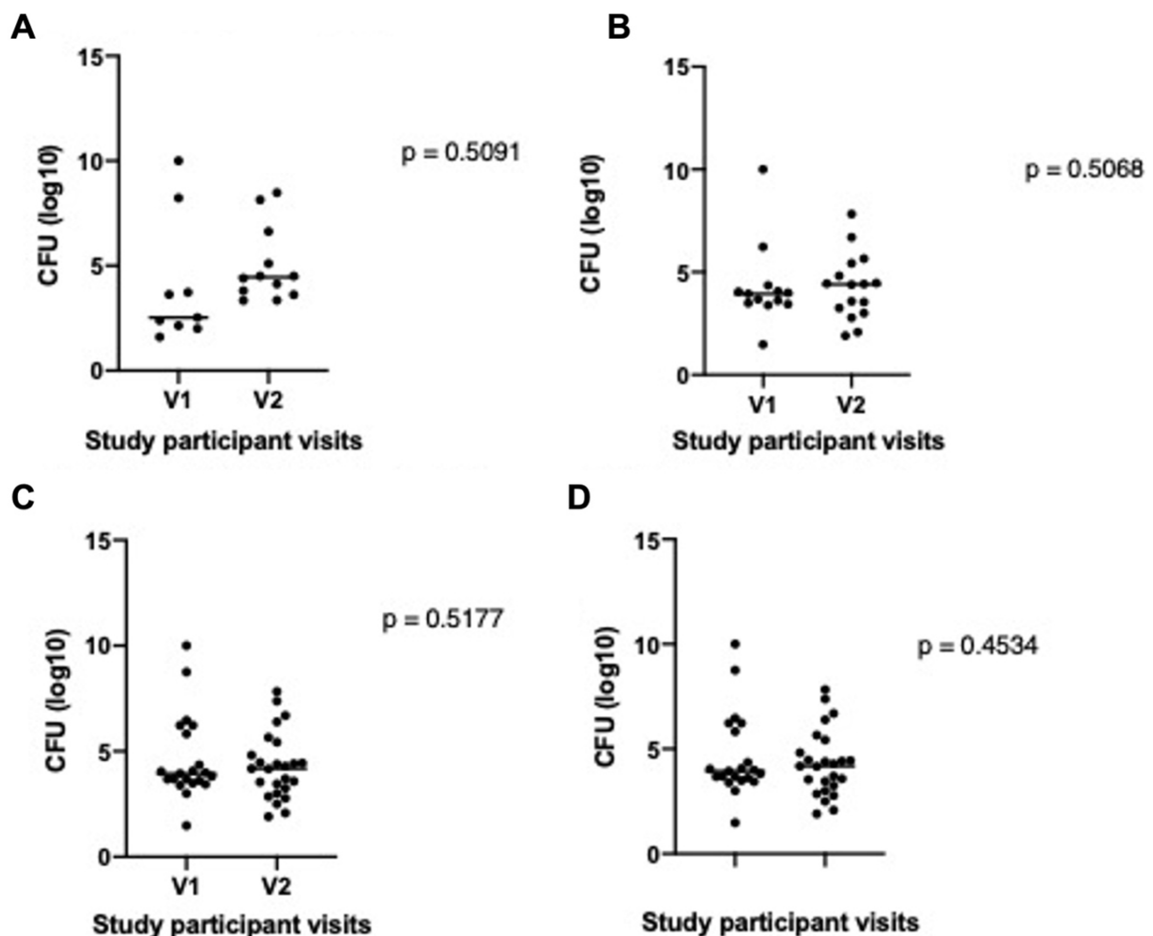


Figure 3. Abundance of coliforms in stool samples. A. Viable counts of coliforms in the placebo group at both sampling timepoints. B. Viable counts of coliforms in the rifaximin-once-daily group at both sampling timepoints. C. Viable counts of coliforms in the rifaximin-twice-daily group at both sampling timepoints. D. Viable counts of coliforms in the combined rifaximin groups at both sampling timepoints.

87/242 (35.9%) of samples demonstrated pin colony growth on ESBL Chromoselect agar after overnight enrichment with LB and Cefotaxime. This comprises of 9 who had ESBL on their initial visit plate, 28 who had ESBL on both visit plates and 22 who had ESBL on their final visit agar plates. 37 of the 121 participants (30.6%) demonstrated growth on their initial sample, thus indicating ESBL-E carriage in the community.

ESBL *E. coli* were isolated from 43/87 (49.4%) of the samples on ESBL agar during the process described above. The study refers to these as resistant *E. coli* (REC). Those *E. coli* grown on UTI Chromoselect agar are referred to as Sensitive *E. coli* (SEC). The remainder of the colonies had morphology consistent with *Klebsiella* and *Citrobacter.* There were also a mixture of pale appearing strains, including *Proteus sp., Morganella sp.* and *Providencia sp.*
E. coli were cultured from the stool of 11/22 (50%) of those individuals who acquired ESBL-E. 2/9 (22.2%) of those who lost ESBL-E and 15/28 (53.5%) of those who retained. ESBL-E. Those who retained ESBL-E had had ESBL-E in both their V1 and V2 samples, hence 30 ESBL *E. coli* from 15 participants.

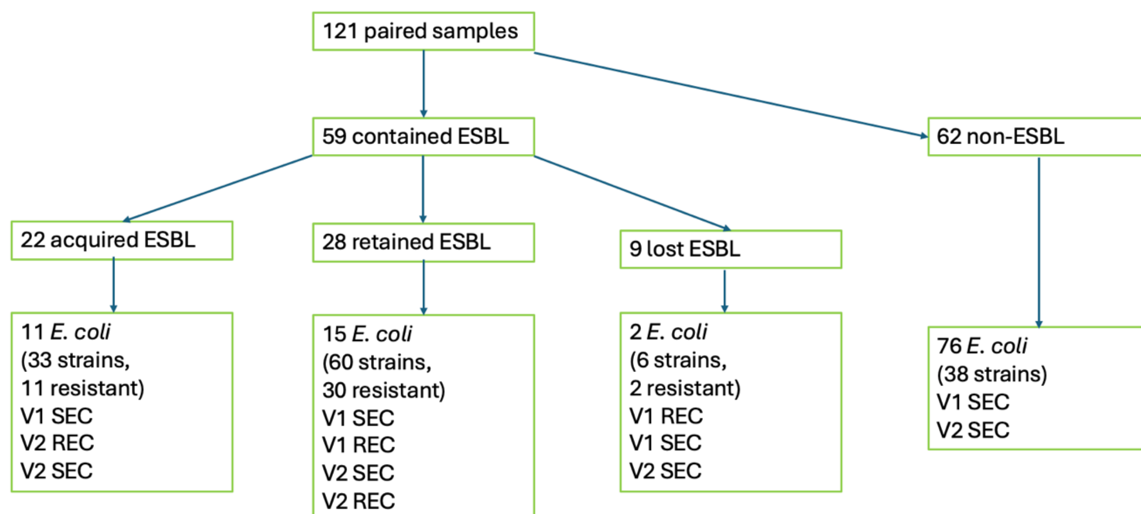


Figure 4. Breakdown of participants to those that acquired, retained, and lost ESBL during PREVENT TD study.

At the end of the 6-week deployment to Kenya, 22/84 (26.5%) of those participants who did not start the study with ESBL-E had acquired ESBL-E. Interestingly, the rate of ESBL-E acquisition was highest in those randomised to placebo prophylaxis, 9/24 (37.5%). This rate is similar to that demonstrated in the COMBAT study, in which 27.8% travellers to East Africa, including Kenya (Arcilla *et al*. 2017) were colonised by ESBL-E.
The ESBL-E acquisition rate decreased with increasing rifaximin prophylaxis dose when compared to placebo (Table 2). Those taking rifaximin 550mg once daily and twice daily, had 7/30 (23.3%, p=0.37) and 6/30 (20%, p=0.22) ESBL-E carriage rates at both time points, respectively. When combining the rifaximin groups, there was an ESBL-E acquisition rate of 13/60 (21.7%, p=0.17). The two rifaximin intervention arms were compared to placebo using Fisher’s exact test.
Statistical significance in the secondary analysis was not reached.

	Acquired ESBL-E	p-value
Placebo	9/24 (37.5%)	--
Rifaximin 550mg once-daily	7/30 (23.3%)	0.37
Rifaximin 550mg twice-daily	6/30 (20.0%)	0.22
Total	22/84 (26.5%)	--
Combined Rifaximin	13/60 (21.7%)	0.17

Table 2. Acquisition of ESBL *E. coli* by intervention in the PREVENT TD study.

In those who began the PREVENT TD study with ESBL-E in their stool (Table 3). 28/37 (75.7%) of them also finished the study with ESBL-E in their stool. We cannot be certain these are the same ESBL-E and not replacement or of the original ESBL-E. However, the persistence of ESBL-E in the gut microbiome across the deployment is of importance. 9/37 (24.3%) lost their ESBL-E during the study. 14/16 (87.5%, p=1.0) of those taking rifaximin 550mg once daily retained their ESBL-E. Half (5/10; p=0.18) of those taking rifaximin 550mg twice daily retained their ESBL-E. Again, this did not reach statistical significance, but is an interesting finding that may be of clinical relevance if replicated in a larger cohort.

	Retained ESBL-E	Lost ESBL-E	p-value
Placebo	9/11 (81.8%)	2/11 (18.2%)	--
Rifaximin 550mg once-daily	14/16 (87.5%)	2/16 (12.5%)	1
Rifaximin 550mg twice-daily	5/10 (50%)	5/10 (50%)	0.18
Total	28/37 (75.7%)	9/37 (24.3%)	--
Combined Rifaximin	19/26 (73.1%)	7/26 (26.9%)	0.69

Table 3. Retention and loss of ESBL by intervention group during PREVENT TD study.

The European Committee Antimicrobial Susceptibility and Testing (EUCAST) produce clinical break point tables to guide the dosage of antibiotic in clinical therapy

and to determine whether an isolate is resistant or susceptible. EUCAST tables v13.1 (EUCAST 2023) with regards to *Enterobacterales* do not give a breakpoint for rifaximin. It also does not give a break point for rifampicin, from which rifaximin is derived. A recent study by Baumert et al (Baumert et al. 2020) examining rifaximin resistance in enteric bacteria used an MIC of 64mg/L or greater to identify resistance, with MICs of 32mg/L or below indicating susceptibility.

EUCAST does not formally set a breakpoint for azithromycin and *Enterobacterales*, but it includes the comment that “isolates with MICs above 16mg/L are likely to have azithromycin resistance mechanisms.” (EUCAST 2023). This value was used as the resistance cut off for strains, with 8mg/L and below indicating susceptibility.
The Clinical and Laboratory Standards Institute (CLSI) subcommittee on Antimicrobial Susceptibility Testing (Weinstein and Lewis 2020) uses a MIC of 32mg/L or above.

The agar dilution susceptibility testing showed that 34 of the 150 (22.7%) strains demonstrated rifaximin resistance with an MIC of 64mg/L and greater.
This included 10/61 (16.4%) of baseline samples, which showed rifaximin resistance. At the conclusion of the study 22 individuals out of 61 (36.0%) had *E. coli* strains that demonstrated rifaximin resistance. 16/51 (31.4%) acquired rifaximin resistance, which is likely related to the selective pressure of rifaximin prophylaxis. 11/16 (68.8%) were taking rifaximin prophylaxis with 5/16 (31.3%, p=1.00) in the placebo arm of the study. 6 (37.5%, p=1.00) were taking twice daily rifaximin prophylaxis and 5 (31.3%, p=1.00) were taking rifaximin once daily.

23 of the 150 (15.3%) strains demonstrated azithromycin resistance with an MIC of 32mg/L and greater. This included 7/61 (11.5%) of baseline samples showed azithromycin resistance. At the conclusion of the study 24 individuals out of 66 (36.4%) had *E. coli* strains that demonstrated azithromycin resistance.

2.3. DISCUSSION

Antibiotic prophylaxis for surgical procedures in wounded personnel is with β-lactam antibiotics such as cephalosporins. This would not be effective in these colonised individuals and could lead to post-operative infection and delayed recovery from injuries.

A PubMed search (29 Sep 2021) with keywords “ESBL” and “rifaximin” provided just 6 results. Rifaximin is effective against *E. coli* thus it may be those strains carrying ESBLs are instead susceptible to rifaximin. Intestinal decolonisation of ESBL-E using rifaximin is possible. Given that rifaximin is non-absorbed and that the intestines are a reservoir of asymptomatic ESBL-E, this seems a reasonable strategy. A retrospective study of ESBL-E decolonisation regimens, in individuals who suffered recurrent ESBL infections in Germany between 2008-2012, compared 2 regimens of colistin and rifaximin. The study demonstrated that colistin was able to decolonise ESBL-E in 3/12 (25%) of patients, whilst rifaximin successfully decolonised 9/15 (60%) (Rieg *et al*. 2015). Rifaximin dosing in Rieg *et al.* was 400mg twice daily for 2-3 weeks, this is a lower dose and shorter duration than the higher dosing of the PREVENT TD study. 3 months post-treatment, 7/13 (54%) of patients were recolonised with ESBL-E. The average age of study participants was 57, which is older than the PREVENT-TD study population. Other factors contributing to their

findings were not elucidated by the paper. They conclude that there is “no clear evidence of significant decolonization efficacy using single-drug treatment with oral non-absorbable antibiotics.”

There was a higher level of rifaximin resistance seen in the study cohort in this rifaximin naïve population, which suggests that the soldiers have been exposed to rifaximin-resistant bacteria, and potentially colonised through poor hygiene practices. Their travel history is not known prior to this study. Not sure what you mean to convey here. Perhaps: The data in this study may not necessarily be generalisable to other populations and destinations.

A further study from 2021 by Kantele (Kantele and Lääveri 2021) studied diarrhoeagenic *E. coli* (DEC) and the rates of antibiotic resistance. The ESBL-E rate was 2.7% amongst DEC *E. coli* strains. The paper also contains a literature review of AMR in DEC. Rifaximin resistance is rarely tested for in studies of ESBL, but the two studies cited by Kantele and Lääveri did specifically test for it and report 0% (Guiral *et al*. 2019) and 16-20% (Ouyang-Latimer *et al*. 2011)
Kantele and Lääveri argue that most episodes of TD will self-resolve and that antibiotics prescriptions for TD should be very much limited.

As was mentioned previously, AMR development has been an issue with past antibiotic prophylaxis. A concern with rifaximin prophylaxis is the development of rifaximin resistance. However, we can conclude that there is no evidence of increased acquisition of ESBL-Enterobacteriaceae in a UK Military population travelling to Kenya taking rifaximin prophylaxis to prevent travellers’ diarrhoea. Antimicrobials are shown to be an independent risk factor, which predisposes travellers to acquiring AMR strains (Kantele *et al*. 2015). A study of 82 *E. coli* isolated from cases of TD demonstrated resistance to Cefotaxime (11/82=13.4%), Ciprofloxacin (18/82=22.0%) and Azithromycin (15/82=18.3%). Rifaximin resistance levels remain low; EAEC and ETEC strains with an MIC > 32μg/ml were not found (Guiral *et al*. 2019).

High stool concentrations of rifaximin can be achieved with a short dosing regimen. In a study of 145 bacterial pathogens from Mexico in the late 1990s, 90% of isolated were inhibited at a concentration of 50μg/ml (Jiang *et al*. 2000). After 3 days of dosing at 800mg/ day, stool concentration reached concentrations of 4,000 – 8,000μg/g. Two dosing regimens were used in the PREVENT TD study; 550mg and 1,110mg/ day. Rifaximin is largely non-absorbed from the gut, with only between 0.01% and 0.4% of the oral dose absorbed from the gut and detectable in plasma (Descombe *et al*. 1994) (Chang *et al*. 2008). This is far above the MICs that are generally observed for microbial isolates (Ponziani *et al*. 2017).
Rifamycins are used in the treatment of a number of serious human diseases, such as infections with methicillin-resistant *Staphylococcus aureus* (MRSA) and tuberculosis (Farrell 2013). The use of rifamycin leads to the development of resistance, and there is a reasonable concern about prolonged use of rifaximin leading to rifamycin resistance. However, there is little evidence for it currently (Calanni *et al*. 2013). Only 2 patients out of 140 (1.4%) who were treated for Hepatic Encephalopathy (HE) for 6 months with rifaximin, developed rifaximin resistant *Clostridium difficile* (Bass *et al*. 2010).

Concerningly, a recent study found that rifaximin use may select for mutations that confer resistance to daptomycin in vancomycin-resistant *Enterococcus faecium* strains (Turner et al. 2023)

Development of rifaximin resistance requires chromosomal mutations, which are vertically transmitted and therefore are not disseminated amongst the intestinal microbiome (Scarpignato and Pelosini 2005). Rifaximin resistant strains are lost from the gut microbiome within weeks of cessation of treatment, suggesting that resistance to rifaximin comes at a fitness cost, allowing susceptible strains to outcompete resistant strains in the absence of selection pressure (De Leo et al. 1986).

In a laboratory setting, *E. coli* can evolve to accumulate rifaximin-resistance in mutations (Ruiz *et al*. 2008). Rifaximin has low levels of resistance selection, but may select highly resistant stable mutants in one step. Development of rifaximin resistance will also lead to rifamycin resistance, which will hamper use of this class of antibiotics in the future.

CHAPTER 3: Analysis of *E. coli* diversity in the PREVENT TD cohort using genome sequencing

3.1. INTRODUCTION

The human colon supports the growth of commensal bacteria, collectively known as the gut microbiota (Katouli 2010). These bacteria exist in a symbiotic relationship with the human host. They receive nutrients from the host, while they in turn, provide benefits to the host e.g. through the production of vitamins and breakdown of complex carbohydrates in the diet (Salyers et al. 1994). Their main function within the large intestine is to protect the mucosa, with structural and metabolic benefits (O'Hara and Shanahan 2006). The intestinal microbiota size is variable depending on species, but usually contains between 10^8 – 10^{12} bacteria per gram of stool (MacFie 2004).

Many factors impact the homeostasis of the gut microbiota, such as dietary changes, physical stress and infectious diseases (Stecher and Hardt 2008). Food has also been implicated as a source of new bacterial pathogens (Rodriguez-Siek *et al*. 2005).

Colonisation resistance describes the mechanisms by which the gut microbiota provides resistance to colonisation by pathogenic microbes (Ducarmon et al. 2019). There are a number of mechanisms that fall under this umbrella term and the exact mechanisms are not clear. A healthy gut microbiome protects against infection by secreting antimicrobial products, maintaining the gut epithelial barrier and host immune activation (Vollaard and Clasener 1994). Observational studies examining the relationship between a healthy gut and the presence of commensal bacteria have progressed to the development of interventional studies (Turnbaugh et al. 2009). *E. coli* can provide a protective function by competing with pathogens for receptors and nutrients within the intestines (Guarner and Malagelada 2003).

Mobile genetic elements are part of a host genome and are passed on during cell division and chromosomal replication (De Oliveira et al. 2020). They include integrative and conjugative elements (ICEs),integrative mobilisable elements (IMEs) and plasmids (Winter et al. 2021). These elements play a significant role in horizontal gene transfer, facilitating the spread of genetic traits such as AMR, virulence factors, and metabolic capabilities among bacterial populations (Frost et al. 2005). Their mobility and ability to integrate into diverse genomic contexts contribute to bacterial adaptability and evolution, posing both challenges and opportunities in microbiology and biotechnology.

Bacterial plasmids are extrachromosomal structures of genetic material (Johnson and Nolan 2009). They contain genetic material for traits including virulence, AMR and metabolism of rare substances (Funnell and Phillips 2004). They are able to self-replicate and be can transmitted between bacteria, which in turn leads to the spread of AMR (Falkow 1975). The earliest research into bacterial plasmids was focused on those responsible for AMR, which were known as R factors (Watanabe 1963). Plasmids are mobile elements and allow spread of AMR genes (Tschäpe 1994), including between different species (Thomas and Nielsen 2005). This process

is known as horizontal gene transfer (Davies and Davies 2010) with the most common pathways being conjugation, transduction and transformation (Soucy et al. 2015).

AMR genes can also be found on chromosomes (Berbers *et al*. 2020). This means they are less likely to be transferred through horizontal gene transfer and their transmission to daughter cells occurs during cell division. This is known as vertical gene transfer (Lawrence 2005). While chromosomally integrated genes are less likely to be transferred, they can be associated with integrative and conjugative elements or be transferred via a process called transformation, in which naked DNA is taken up from the environment by bacteria and integrated into their chromosome (McInnes et al. 2020).

While plasmids are important for the dissemination of AMR genes, they are difficult to reconstruct using second generation, “short read”, sequencing data (Arredondo-Alonso *et al*. 2017).

A common commensal gastrointestinal tract bacterium responsible for many intestinal and extraintestinal infections is *E. coli* (Bailey et al. 2006), which is a Gram-negative, facultative anaerobic coliform bacillus (Tenaillon *et al*. 2010). It is commonly found in the intestines of vertebrates. It can be responsible for both intestinal and extra-intestinal infections. *E. coli* is a well-studied bacteria and a large number of genome sequences are publicly available and it is frequently used as a surrogate to determine resistance levels in the microbiome (Brisola et al. 2019).

Multi Locus Sequence Typing (MLST) is a method of typing bacterial strains based on sequences of multiple housekeeping genes which are genes with a low rate of change, that are not under rapid evolutionary selection. MLST compares allelic differences rather than sequences. A single MLST scheme based on one species may not be able to assign different organisms to other species. Large specialist databases incorporating multiple core genome MLST schemes, such as Enterobase (Achtman et al. 2022), can assign a submitted genome to a number of different enterobacterial species (Maiden et al. 2013).

E. coli genotypes can be further grouped into clonal complexes. These are close genetic variants belonging to a specific ST, which comprise genetically similar isolates and exhibit small changes in the genetic sequences that define the MLST (Feil *et al*. 2004).

3.1.1. POPULATION STRUCTURE OF *E. coli*

E. coli is a highly diverse species and different phylotypes have been identified that form major clusters within species. To facilitate the assignment of strains to phylogroups, a triplex PCR method was described by Clermont and colleagues (Clermont et al. 2000). At the time, pathogenic and commensal *E. coli* were collectively classified into 4 different phylogroups: A, B1, B2 and D (Herzer *et al*. 1990). The phylotypes A and B1 are mainly commensal strains from human and animal large intestines, and environmental samples (Rijavec *et al*. 2008). These strains do not usually contain any virulence factors and thus have low pathogenic potential (Johnson *et al*. 2001). However, phylogenetic group B2 and D consist of

pathogenic strains and carry VF (Bingen *et al*. 1998). They are also commonly found in healthy gut microbiomes. Further advances in sequencing over the years have led to the definition of 7 phylotypes (Clermont *et al*. 2013).

Pathogenic *E. coli* strains are associated with specific phylogroups and STs, which correlate with their pathotypes. For example, extraintestinal pathogenic *E. coli* (ExPEC), including uropathogenic *E. coli* (UPEC), is predominantly found in phylogroup B2 and sometimes D. In contrast, shiga toxin-producing *E. coli* (STEC), responsible for severe gastrointestinal infections, is often associated with phylogroup B1. These relationships highlight the genetic basis for the adaptability of *E. coli* to diverse pathogenic lifestyles, underscoring the importance of phylogenetic studies for understanding and managing these infections (Denamur et al. 2021).

E. coli ST69 (along with ST73, ST95 and ST131) are responsible for a high proportion of *E. coli* urinary tract infections (UTIs) and bacteraemia (Doumith *et al*. 2015). In a study of diarrhoeagenic *E. coli* (DEC) from Southeastern China from 2009-2011, isolates of ST69 were highly resistant to the majority of antimicrobials (Chen *et al*. 2014).
ST73 is an extra-intestinal pathogenic *E. coli* (ExPEC), a strain that asymptomatically colonises the bowel, but can be pathogenic outside the gut (Foxman 2010). ST73 is in the same phylogroup as ST131 and has increased in prevalence recently (Alhashash et al. 2016).
ST131 is a clinically important, wide spread pathogenic lineage that typically displays resistance to ESBL (Hastak *et al*. 2020).
ST10 is a common commensal *E. coli*, which was present in all human and food-animal sources from overlapping geographical areas between 2005-2010 in Canada (Manges *et al*. 2015).
ST34 is another common commensal *E. coli*. It has not been previously reported as an ESBL producing strain (Pietsch *et al*. 2017).

The term virulence factors (VF) applies to gene products, which enable a pathogenic organism to cause tissue damage (Chen *et al*. 2005). Traditionally, VFs referred to secreted proteins, such as toxins, cell surface structures and enzymes, which directly cause disease. It has since been recognised that other genes encoding mechanisms indirectly involved in pathogenesis are equally important (Brogden 2000).

E. coli has a wide range of VF that contribute to the different infections caused by this pathogen and some of the most prominent will be discussed below. *Eae* is an *E. coli* -attaching and -effacing gene. Strains containing *eae* are heterogenous in respect to their virulence determinants (Schmidt *et al*. 1994). In some instances, it plays an important role in pathogenesis of Shiga Toxin-producing *E. coli* (STEC). This is a pathogen associated with foodborne gastroenteritis (Bryan et al. 2015). The *eae* gene encodes an adherence protein, intimin. Prevalence of *eae* in STEC strains range from 52.5% (Byrne *et al*. 2014) to 90.8% of clinical STEC strains (Hoang Minh *et al*. 2015). Intimin is also an important VF of other bacteria, such as EPEC and *Citrobacter rodentium* (Lacher et al. 2006). It plays a critical role in intestinal colonisation (Yang *et al*. 2020). Other VF are also required for pathogenicity, such as the plasmid-carried enterohemolysin gene, *exhA* (Lorenz *et al*. 2013).

Shiga toxin (Stx) virulence factor is considered the primary VF responsible of Shiga Toxin-producing *E. coli* (STEC) causing Haemolytic Uraemic Syndrome (HUS) and Haemorrhagic Colitis (Lee and Tesh 2019).

The *ipaD* gene is one of 3 invasion plasmid antigens involved in the expression of invasive phenotypes in all species of *Shigella* and EIEC (Venkatesan et al. 1989). DNA probes for these genes accurately indicate invasive phenotypes (Venkatesan *et al*. 1988). EIEC and *Shigella sp.* cause bacillary dysentery (shigellosis) with systemic illness (Belotserkovsky and Sansonetti 2018) EIEC does not produce toxins, but cause mechanical destruction to the intestinal wall (Dong *et al*. 2020). It uses a protein transport device called a Type 3 Secretion System, which requires 3 protein translocators, IpaB, IpaC and IpaD (Roehrich *et al*. 2013). IpaD is hydrophilic (Espina *et al*. 2006) whilst the other 2 are hydrophobic. IpaD most likely serves as a scaffold for the hydrophobic proteins, which then form a pore in the in the host cell membrane (Veenendaal *et al*. 2007).

The genes *aat* and *aggR* are plasmid borne genes that are typical for EAEC (Huang *et al*. 2007). Most EAEC strains harbour the transcriptional factor *AggR*, which then activates several other factors (Nataro *et al*. 1994). The Aggregative Adherence Fimbriae (*aaf*) genes are controlled by the *aggR* gene (Bernier et al. 2002). AAFs are required for adherence to human intestinal cells, in order to allow opening of epithelial tight cell junctions and cytokine release (Strauman *et al*. 2010).
A study of an EAEC strain O104:H4 that was responsible for over 400 cases of diarrhoea with 22% developing HUS in Germany in 2011 (Frank *et al*. 2011). This EAEC was unusual in that it contained prophage containing the gene for Shiga toxin (Muniesa et al. 2012). Laboratory deletion of the *aggR* gene in this strain significantly reduced bacterial adherence and translocation (Boisen *et al*. 2019).
EAEC are an important cause of diarrhoea worldwide. However, the heterogeneity of EAEC gene profiles makes establishing pathogenicity difficult (Bamidele et al. 2019). EAEC are defined by their adherence to HEp-2 cells in an “aggregative adherence” pattern (Nataro *et al*. 1994). However, this adhesion assay is labour intensive and requires expertise to interpret the results (Nataro 2005). Thus a PCR method was developed to target the high molecular weight plasmid on which a number of the virulence genes are located (Baudry *et al*. 1990). EAEC can cause persistent diarrhoea with 20-30% of cases lasting longer than 14 days (Čobeljić *et al*. 1996).

Some *E. coli* strains produce colibactin, which is a genotoxic compound, inducing DNA double strand breaks, which, in turn, can lead to chromosomal aberrations and cell-cycle arrest (Faïs *et al*. 2018). Biological studies have shown that colibactin-producing bacteria have anti-inflammatory, antibiotic, anti-inflammatory and analgesic effects (Olier et al. 2012; Vizcaino et al. 2014; Pérez-Berezo et al. 2017). *E. coli* strains producing colibactin are overrepresented in colorectal cancer samples (Bonnet *et al*. 2014). However, there is no clear causative mechanism and a number of beneficial effects are also seen in colibactin producing *E. coli.* A common probiotic strain *E. coli* Nissile 1917, which has uses in treating intestinal disorders also produces colibactin (Wassenaar 2016).

3.2 MATERIALS AND METHODS

3.2.1. STRAIN PREPARATION

Strains were isolated using a protocol, which involved taking a single *E. coli* colony from the ESBL Chromoselect agar plate and corresponding UTI Chromoselect agar. This was streaked out onto a fresh agar plate, which was incubated overnight at 37ºC. A sterile loop was then used to take all bacterial culture off the plate and mixed into tubes containing beads and cryopreservant liquid, as provided by MicrobesNG (Birmingham, UK). Sequencing was performed by MicrobesNG on the Illumina sequencing platform (Illumina Inc., San Diego, CA, USA) (www.illumina.com) and produced 2x250bp paired-end reads.

MicrobesNG then analysed the data through their standard analysis pipeline. The closest available reference genome is identified using Kraken (Wood and Salzberg 2014) and map the reads to this using BWA-mem (Li and Durbin 2009) to assess the quality of the data. De novo assemblies of the reads were then performed using SPAdes (Bankevich et al. 2012) and these assemblies were then made available for download in FASTA format.

All bioinformatics was performed on the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) (Connor *et al*. 2016) server of the University of Birmingham.

3.2.2. SEQUENCE TYPING

Kraken (version 1.1.1) and MLST (version 2.19.0) were used for sequence typing against ".fasta." sequencing outputs. EzClermont (version 0.6.3) was used for Clermont typing of the *E. coli* sequences (Maiden 2006; Clermont et al. 2013; Wood and Salzberg 2014).

Phylogenetic analysis using core genome alignment was performed using the command line tools, Parsnp (version 1.5.6) (Treangen *et al*. 2014). The genome of *E. coli* K12 MG1655, which is a common laboratory reference sample was used as a reference isolate (Blattner *et al*. 1997).
Raw data was uploaded to iTOL to construct and visualise phylogenetic trees.

SNP distances were calculated using snp-dists (version 0.8.2) and snippy (version 4.6.0).

3.2.3. VIRULENCE FACTORS

Virulence factors were identified using Abricate (version 1.0.1) (T. Seeman https://github.com/tseemann/abricate) Virulence Factor DataBase (VFDB) (Chen *et al*. 2016) and Ecoli vf (https://github.com/phac-nml/ecoli_vf) , which is a repository containing VF from *E. coli* in VFDB and supplemented with additional VF from the literature.

A refined database using VF associated with Intestinal Pathogenic *E. coli* (IPEC) associated VF was created (Robins-Browne *et al*. 2016). Table 4.

Pathotype	Defining marker	Essential virulence determinant(s)	Location of essential virulence determinant(s)	Major diagnostic target(s) for PCR	Other diagnostic target(s)
EPEC	LEE PAI	LEE PAI	Pathogenicity island	*eae*	*bfpA*[a]
EIEC/Shigella	pINV	pINV	Plasmid	*ipaH*	Other *ipa* genes
ETEC	ST or LT	ST and/or LT plus colonisation factors	Plasmid; transposon	*elt, est*	
EHEC	Shiga toxin	Shiga toxin 1 and/or 2	Prophages	*stx1, stx2*	*eae*[a], *ehxA*[a]
EAEC	pAA; aggregative adhesion	Not known	Plasmid (probably); possibly others	*aggR, aatA, aaiC*	
DAEC	Afa/Dr adhesins	Not known	Not known	Afa/Dr adhesins[b]	
AIEC	Adherent-invasive phenotype	Not known	Not known	none	none

aaiC, gene for a secreted protein of enteroaggregative E. coli; aatA, gene for a transporter protein of enteroaggregative E. coli; Afa, afimbrial adhesin; aggR, gene for a transcriptional regulator; AIEC, adherent-invasive E. coli; bfpA, gene for a structural protein of bundle-forming pili; DAEC, diffusely-adherent E. coli; EAEC, enteroaggregative E. coli; EIEC, enteroinvasive E. coli; elt, gene for heat-labile enterotoxin; EPEC, enteropathogenic E. coli; est, gene for heat-stable enterotoxin; ETEC, enterotoxigenic E. coli; ipaH, gene for a type 3-secreted effector protein of enteroinvasive E. coli and Shigella; LEE PAI, locus of enterocyte effacement pathogenicity island; LT, heat-labile enterotoxin; pAA, virulence plasmid of enteroaggregative E. coli; pINV, virulence plasmid of enteroinvasive E. coli and Shigella; ST, heat-stable enterotoxin.

[a]Not present in all strains.

[b]These are under review following concerns about specificity.

Table 4. IPEC associated VF for E. coli

3.2.4. PLASMIDS

To identify plasmids within the genome sequences, a search was performed using the PlasmidFinder database (Carattoli *et al*. 2014) with the Abricate bioinformatics tool.

3.2.5. ANTIMICROBIAL RESISTANCE

The bioinformatic tool Abricate (version 1.0.1) (T. Seeman https://github.com/tseemann/abricate) using National Centre for BioInformatics (NCBI) AMRFinderPlus (Feldgarden *et al*. 2019) and ResFinder databases.

3.3. RESULTS

Corresponding strains that grew on ESBL Chromoselect agar and UTI Chromoselect agar from the same individual (2 strains at each time point) were isolated and sent for genomic sequencing, along with 76 *E. coli* strains from 38 individuals, who did not have ESBL-E in either their initial or final visit stool samples. In total 177 strains were sent for sequencing. There were 40 resistant *E. coli* (REC) and 137 sensitive *E. coli* (SEC). Though it is also possible to grow resistant *E. coli* on the UTI Chromoselect agar.

3.3.1. SEQUENCE TYPING

Analysis with Kraken and MLST confirmed 160/177 (90.4%) were *E. coli* sequences, comprised of 122 sensitive strains and 38 strains isolated from ESBL-selective agar. This indicated that there had been some mis-identification on the UTI Chromoselect agar, as the following bacteria were recovered in the genome sequence project:

- *Salmonella enterica* (3)
- *Hafnia paralvei* (5)
- *Citrobacter freundii (4)*
- *Staphylococcus epidermidis* (1)
- *Enterobacter cloacae (1)*
- *Escherichia marmotae* (3)

Given it is also an *Escherichia,* Parsnp and iTOL were used to confirm the relatedness of *E. marmotae* and the PREVENT TD cohort of *E. coli* strains (Fig. 5). This analysis confirmed that the *E. marmotae* strains are distinct from *E. coli* and were excluded from further analysis. The branch length fof *E. marmotae* from phylogenetic analysis is 2.213 compared to that of *E. fergusonii* (at 0.966). This indicates that *E. marmotae* is more distantly related to *E. coli* than other common *Escherichia.*

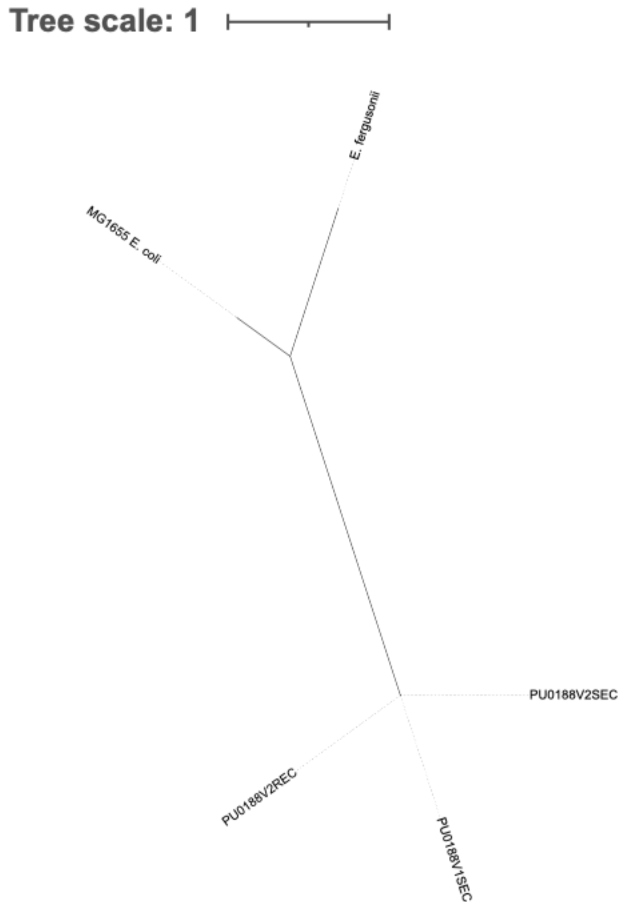


Figure 5. Phylogenetic tree of E. marmotae compared to E. coli and E. fergusonii.

The *E. marmotae* genome sequence was analysed with ClermontTyper (via the web interface http://clermontyping.iame-research.center/) and identified as CladeV *Escherichia*. The *E. marmotae* was phenotypically ESBL.

MLST identified broad range of *E. coli* STs (Fig. 6). The most common was ST69 (31/160 = 19.4%), ST10 (17/160 = 10.6%), ST540 (11/160 = 6.9%) and ST34 (10/160 = 6.3%).

The 160 *E. coli* sequences were analysed with EzClermont (Waters *et al*. 2019) and we found 6 different phylotypes. The most frequent was A (74/160 = 46.3%) then D (40/160 = 25%), B2 (18/160 = 11.3%), B1 (17/160 = 10.6%), F (9/160 = 5.6%) and E (2/160, 1.25%).

In this cohort of *E. coli* from the PREVENT TD study stool samples, ST69, 13/31 (41.9%) were phenotypically ESBL resistant. As were 0% (0/5) of ST 73, 23.5% (4/17) of ST 10 and 60% (6/10) of ST34.
There were ST73 in 5 isolates: PU0006V1SEC1, PU0006V2SEC1, PU0082V2SEC1, PU0102V1SEC1 and PU0191V1SEC1. These patients were well throughout the deployment and randomised to rifaximin twice daily, with the exception of PU0006, which was allocated to the Placebo study arm). There were no ST 131 identified in the strains.

The already identified ST34, ST43, ST48, ST695, ST1303 can be grouped with ST10 into the ST10 Clonal Complex (ST10CC). As described above, ST10 is a common commensal *E. coli.*

This is an expected finding as phylotype A is commensal, however B2 and D are pathogenic strains. Though the individuals were well at the time of sample collection.

E. coli SEQUENCE TYPE	Total
10	12
34	10
43	4
48	2
58	1
59	2
69	20
73	1
182	2
216	1
295	1
361	2
363	1
457	1
540	1
609	3
648	2
695	2
906	1
1139	1
1196	2
1303	2
1304	1
1431	1
2165	1
2795	4
3076	1
3672	2

CLERMONT TYPE	Total
A	50
B1	11
B2	4
D	20
E	2
F	5

Figure 6. E. coli multi locus sequence typing (MLST) and Clermont typing for PREVENT TD samples.

3.3.2. PHYLOGENETICS

Following an initial analysis and removal of the non *E. coli* sequences, a core genome phylogenetic tree was produced using Parsnp (Fig 7). Parsnp does not routinely calculate main node confidences. Rather, it focused on building a maximum likelihood tree. Bootstrap percentages require repeat sampling of the data to generate and the main focus is on speed.

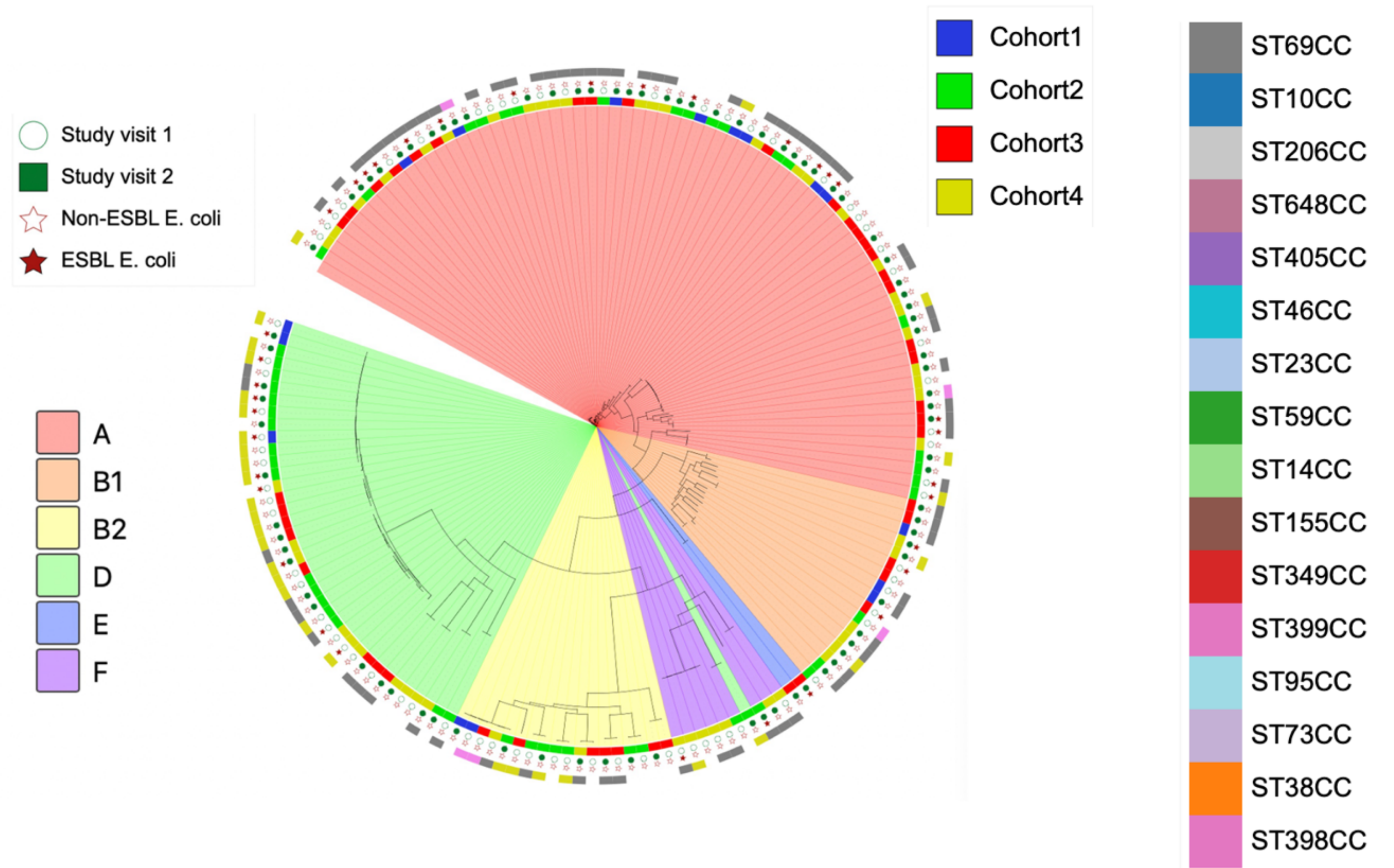


Figure 7. Phylogenetic tree of E. coli from PREVENT TD study. CC: clonal complex.

A tree of only the strains isolated on the ESBL Chromoselect medium was constructed (Fig 8). It demonstrates the similarities in the resistant *E. coli* strains between 4 individuals from 2 different cohorts. These strains persisted for the duration of the study.
Furthermore, 2 other individuals (PU0206 and PU0210) who were also from the same cohort appear to have transmitted their ESBL *E. coli*, with PU0210 having been colonised with a different ESBL-EC at the beginning of the study and PU0206 being colonised with a different ESBL-EC at the end of the study.

REC of those individuals that acquired ESBL-E during their deployment were analysed using Parsnp and iTOL. This demonstrated that 6 of the strains were similar (Fig. 8). Their study participant numbering indicated that they were from different time points, thus different deployments to Kenya. This suggests environmental contamination with ESBL-E in Kenya. As environmental sampling and subsequent resistance testing is not routinely performed in BATUK, Kenya, it is not possible to identify this source. This is an area of further study that will be explored during subsequent work. Common sources of ESBL *E. coli* may be food washed with contaminated water and shared bathroom facilities (Hooban et al. 2021).

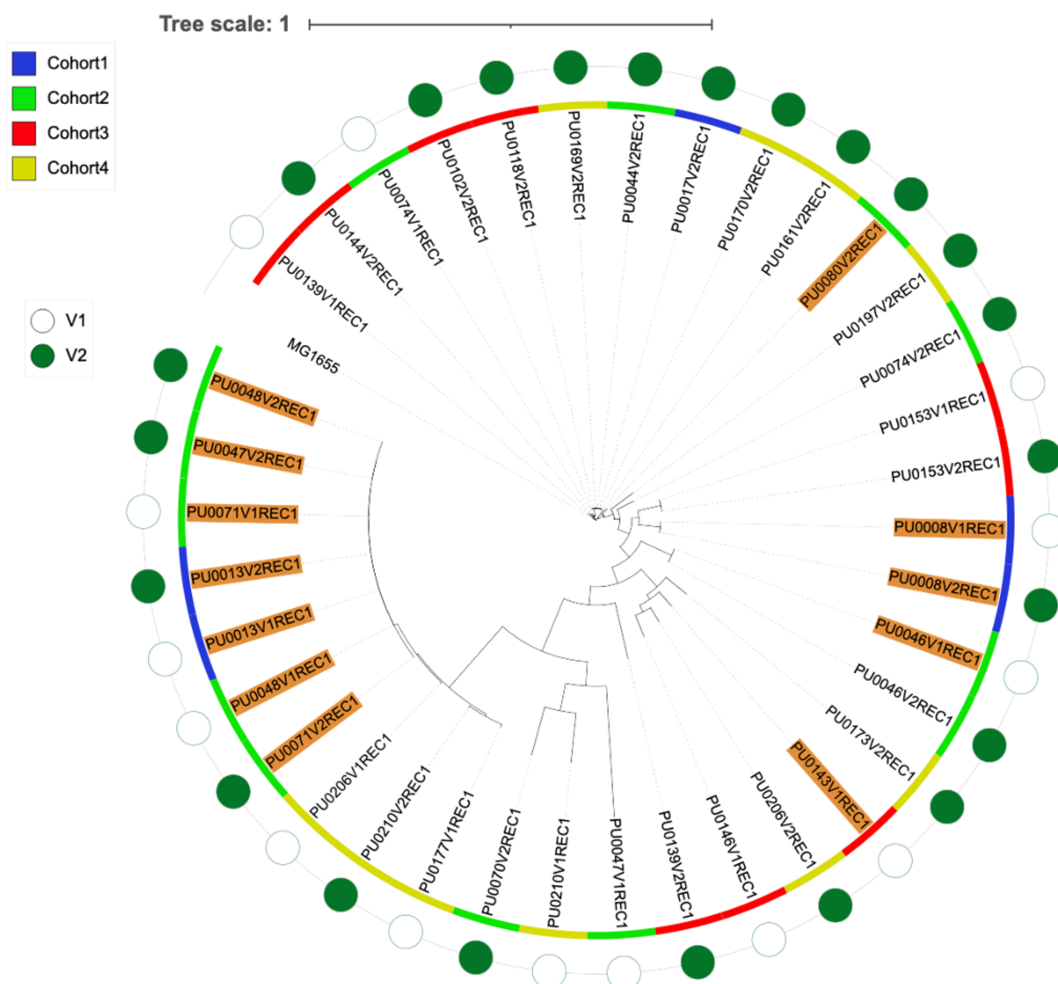


Figure 8. Phylogenetic tree of *E. coli* isolated from ESBL ChromoSelect agar during PREVENT TD study. Participant codes (PU) are included between the branches and the labelling. The highlighted PUs indicate those *E. coli* containing the AMR gene bla-CTX-M-15.

3.3.3. VIRULENCE FACTORS

An initial search using VFDB and Ecoli_vf database for the 160 *E. coli* genomic sequences, produced 29,254 matches with recognised virulence factors (average 181.21 per strain, range 107 – 276.) A search with the refined IPEC associated VF database (Robins-Browne *et al*. 2016) identified 111 results in total.

70 of the 90 (77.7%) *E. coli* sequenced carried the *eaeH* (GenBank accession number P19809.2) VF, with one strain having *eae* (PU0203_V1_SEC1).
EaeH is a highly conserved surface protein associated with EPEC & ETEC (Sheikh *et al*. 2014). In vitro, EaeH expressing *E. coli* have been shown to coat the lining of intestinal epithelial cells. This would then ordinarily lead to the delivery of heat labile toxins (Roy *et al*. 2011). Interestingly, studies have shown EaeH expression is increased following epithelial cell contact (Kansal *et al*. 2013).

27 of the 90 (30%) *E. coli* sequenced showed *ipaD*, a VF associated with EIEC. Neither *ipaB* or *ipaC* were detected in any of these *E. coli* strains. A complex of ipaB, ipaC and ipaD are necessary for entry into epithelial cells (De Geyter et al. 2000).

13/90 (14.4%) of sequenced *E. coli* had VFs associated with EAEC. 10 strains showed *aatA* and 3 strains carried a combination of *aatB*, *aatC*, *aatD* and *aatP*. Interestingly, these four genes always appeared together in this cohort. 3 other strains had the *aggR* gene.

Afa adhesin genes (GenBank accession numbers AZ935556 – AZ935604) were found in a single *E. coli* strain, PU0196_V1_SEC1. These are associated with DAEC. Given it was in the V1 sample, the participant was colonised with this particular *E. coli* strain when they arrived in Kenya. They were well at the time and for the duration of the study. Although, Afa expressing *E. coli* cause diarrhoea in children up to the age of 5, their role in diarrhoea in adults is less certain, and strains carrying this gene are often present in asymptomatic intestinal microbiota strains (Le Bouguénec and Servin 2006). The mechanisms in pathogenicity and pregnancy related complications is also well-established (Servin 2014).
The *afa* gene encodes afimbrial adhesins, which allow Afa expressing *E. coli* to attach to target host cells and resist clearance by intestinal peristalsis (Kline *et al*. 2009). This allows initiation of mechanisms which dramatically impair host cell function (Hayward *et al*. 2006). *E. coli* strain, PU0196_V1_SEC1 expressed *afaA*, *afaB-I*, *afaC*-I, *afaD* and *afaE-I*.

The VF *exhA* was not identified in any of the *E. coli* strains sequenced. Reassuringly, there also no evidence of shiga toxin (Stx) virulence factor.

The genes encoding the biosynthetic machinery of toxin colibactin (*clbA*) was found in one genome (PU0102_V1_SEC1). Participant PU0102 was randomised to rifaximin 550mg twice daily.

In summary, the most common virulence factor identified was *eaeH* (77%), which is associated with EPEC & ETEC. The 2nd most common VF was *ipa* gene (27/90 = 30%) associated with EIEC and 3rd most common was *aat* genes (13/90 = 14.4%). There were also single instances of *afa* adhesin genes and *clbA*. Reassuringly, there

was no evidence of *stx* genes in the *E. coli* strains that had their genomes sequenced here.

Despite the high levels of VF, there was low levels of clinically apparent TD. 17 of the study participants suffered TD, unfortunately diarrhoeal stool samples from the time are not available, given the difficulties of collecting and storing samples commonly experienced. 8 of these individuals had paired samples, for which I was able to recover *E. coli.*

3.3.4. ANTIMICROBIAL RESISTANCE GENES

The 160 *E. coli* genomic sequences (comprised of 122 sensitive strains isolated from UTI Chromoselect medium and 38 strains isolated from ESBL-selective agar) provided 670 “hits” for AMR genes. All phenotypically ESBL-producing *E. coli* strains expressed either cephalosporin or β-lactam resistance genes

This Abricate data was combined with Parsnp and iTOL then annotated with a heat map to display the presence of AMR genes (Fig 9). 18 out of 38 phenotypically ESBL-positive strains contained a recognised gene causing this phenotype.

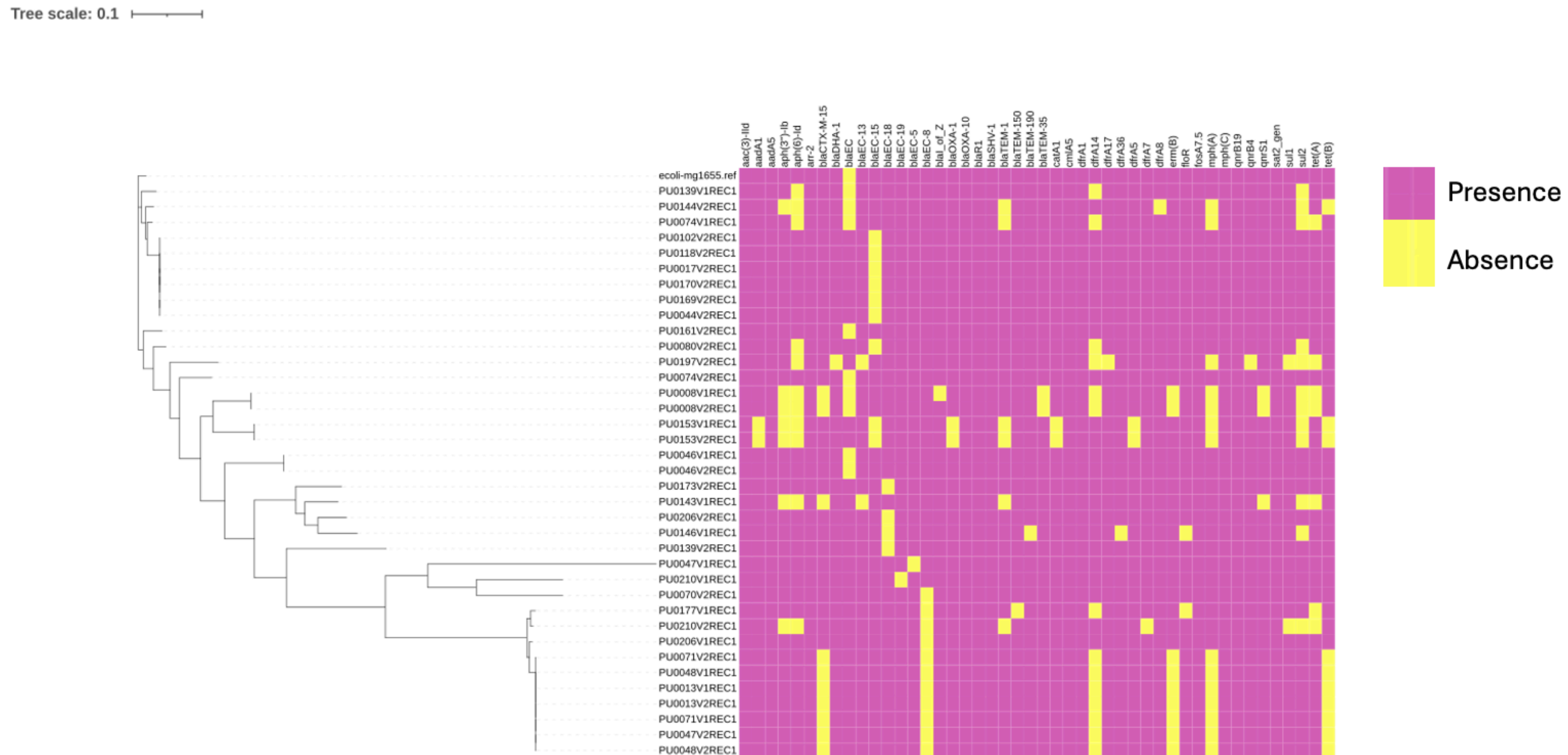


Figure 9. Antimicrobial resistance genes for *E. coli* isolated on ESBL Chromoselect medium from PREVENT TD study.

A common EBSL gene is blaCTX-M-15. This was found in 10 (26.3%) of the *E. coli* strains isolated from ESBL-selective media. Seven of the ST69 isolates have the gene inserted at the same chromosomal position. These resistant *E. coli* samples are PU0013V1REC1, PU0013V2REC1, PU0047V2REC1, PU0048V1REC1, PU0048V2REC1, PU0071V1REC1 and PU0071V2REC1.
PU0073V1REC1 is also an ST69 strain, but its CTX-M-15 gene is in a different context, it has probably acquired the CTX-M-15 gene via a different mobile genetic element. The CTX-M-15 gene is located on a plasmid in a region that is associated with IS26. IS26 insertion sequences plays a pivotal role in spreading AMR genes amongst Gram negative bacteria (Harmer et al. 2014).
PU0008V1REC1 and PU0008V2REC1 are both ST361 strains and have CTX-M-15 in the same position, which is consistent with them being the same strain that has persisted in this individual during the deployment. The context of the AMR gene suggests it is located on a plasmid. When the sequence of the contig that includes the CTX-M-15 gene is queried using GenBank (Benson *et al*. 2013) it reveals it most closely resembles plasmids found in an *E. coli* ST69 from raw milk and cheese in Egypt (Hammad *et al*. 2019). It is possible this individual had travelled to Egypt prior to their deployment to BATUK, but also possible that sampling bias means these strains are present across Africa, or globally, but have not been sequenced. As explained previously, travel history was not information that was captured during the study, but in retrospect would have been very useful.
PU0143V1REC1 is ST1431 and contains CTX-M-15 in another context. This also appears to be on a plasmid, which most closely matches a plasmid from an *E. coli* isolated in Nigeria (Fortini *et al*. 2015).

Two strains had genes not expected to cause ESBL resistance, blaDHA (PU0197V2REC1) and blaOXA (PU0153V2REC1) (Barguigua *et al*. 2013). To confirm these strains had an ESBL phenotype, they were recultured in LB-cefotaxime and streaked onto ESBL Chromoselect medium. Growth was seen on the plates.
BlaDHA-1 is a plasmid mediated cephalosporinase (Barnaud *et al*. 1998). It confers resistance to oxyimino-cephalosporins, which include cefotaxime and ceftazidime. It was first identified in 1992 in clinical isolates of *Salmonella enteritidis* from Saudi Arabia (Gaillot *et al*. 1997).
PU0153V2REC1 contains blaOXA-1, which is not supposed to confer ESBL resistance. Some penicillinases are variable in their action. OXA-1 is a poorly inhibited penicillinase (Sugumar *et al*. 2014). In a study of 293 ESBL-EC from bacteraemias, co-carriage of blaOXA-1 was associated with blaTEM (Livermore *et al*. 2019).
This same strain also contains blaTEM. It was unusual in comparison to other blaTEM in the dataset, in that it had a contig coverage of 847.2, whilst the other resistance genes in the genome have a coverage value of 50-100. This suggests that blaTEM is in a high copy number plasmid or has undergone gene amplification This could be significant, as it is known that increasing number of blaTEM copies can lead to unexpected, expanded phenotypes (Hubbard *et al*. 2020). It is also possible that the combination of blaOXY and amplification of blaTEM may lead to a strain becoming phenotypically resistant to cephalosporins, but further work would be required.
It is perhaps notable that the blaTEM containing contig in PU0153V2REC1 is located in a small transposon (Tn2), which in itself is in a small plasmid. The insertion of Tn2

appears to have interupted the gene that regulates plasmid copy number. This results in a high copy number that appears to confer increased resistance profile (Moran and Hall 2017).

All of the strains demonstrate a blaEC (UniProtKB, A0A3T4J2R6) gene. This is a serine β-lactamase with substrate specificity for cephalosporins. It is an outer membrane bound to the periplasmic space and a member of the class-C β-lactamase family (Jain et al. 2021). It is associated with resistance to cephalosporin (Wu et al. 2020).
blaEC is not a familiar ESBL gene, thus repeat laboratory testing demonstrated that those 16/20 (80%) without traditional ESBL AMR genes were phenotypically EBSL. The other 4 grew *Klebsiella* and a pale colony.

3.3.4.1 AZITHROMYCIN RESISTANCE GENES

Enrichment and selection for azithromycin-resistant strains was not conducted as part of this study, in the way that enrichment for strains producing ESBLs was performed. Regardless, 44/160 of all the *E. coli* had *mph(A)* and a further one had *mph(C)*, which means a total of 45/160 (28.1%).
Of the ESBL resistant *E. coli* strains, 14/38 (36.8%) demonstrated *mph(A)* indicating potential for macrolide resistance. This would be a further area of research, which could influence antimicrobial treatment policies in military personnel.

3.3.5 PLASMIDS

Similarly to the findings of the CTX-M-15 genes on the ST69 *E. coli,* there were 10 pairs (one grown on ESBL Chromoselect agar, the other on non-selective agar) of *E. coli* strains at the same time point. They have the same plasmid configuration.

When compared using snp-dists and snippy I was able to produce a SNP distance matrix (Fig 10). The values of SNP distances were found to be in the hundreds, rather than below 40 indicate the strains are not clones, but hold the same mobile genetic elements.

snp-dists 0.8.2	PU0046V2REC1	PU0046V2SEC1	PU0047V1REC1	PU0047V1SEC1	PU0048V1REC1	PU0048V1SEC1	PU0048V2REC1	PU0048V2SEC1	PU0071V2REC1	PU0071V2SEC1	PU0139V1REC1	PU0139V1SEC1	PU0153V2REC1	PU0153V2SEC1	PU0161V2REC1	PU0161V2SEC1	PU0197V2REC1	PU0197V2SEC1	PU0210V1REC1	PU0210V1SEC1
PU0046V2REC1	0	334	10194	10720	9373	9901	9408	9993	9777	9813	4783	4702	4720	4006	4255	4348	4602	4550	9878	9516
PU0046V2SEC1	334	0	10268	10822	9418	9962	9469	10074	9831	9883	4798	4752	4745	4018	4249	4330	4616	4535	9957	9588
PU0047V1REC1	10194	10268	0	320	9899	10269	9878	10356	10169	10170	10109	10055	10566	9334	8504	8719	9408	9260	8856	8591
PU0047V1SEC1	10720	10822	320	0	10329	10838	10309	10909	10690	10730	10608	10519	11019	9554	8737	8961	9771	9583	9246	8989
PU0048V1REC1	9373	9418	9899	10329	0	402	385	414	425	412	9054	8959	9656	8386	7364	7557	8374	8231	8357	8158
PU0048V1SEC1	9901	9962	10269	10838	402	0	310	312	302	298	9505	9459	10236	8747	7577	7789	8721	8537	8752	8550
PU0048V2REC1	9408	9469	9878	10309	385	310	0	325	294	314	9042	9001	9724	8483	7379	7580	8394	8252	8370	8206
PU0048V2SEC1	9993	10074	10356	10909	414	312	325	0	287	329	9572	9473	10319	8756	7639	7814	8779	8603	8864	8680
PU0071V2REC1	9777	9831	10169	10690	425	302	294	287	0	308	9384	9328	10068	8663	7567	7737	8662	8494	8679	8471
PU0071V2SEC1	9813	9883	10170	10730	412	298	314	329	308	0	9427	9358	10172	8697	7577	7764	8672	8498	8708	8514
PU0139V1REC1	4783	4798	10109	10608	9054	9505	9042	9572	9384	9427	0	351	4369	3755	1673	1745	3455	3381	9343	9098
PU0139V1SEC1	4702	4752	10055	10519	8959	9459	9001	9473	9328	9358	351	0	4329	3734	1646	1742	3424	3370	9285	8980
PU0153V2REC1	4720	4745	10566	11019	9656	10236	9724	10319	10068	10172	4369	4329	0	347	3577	3719	4190	4166	9965	9623
PU0153V2SEC1	4006	4018	9334	9554	8386	8747	8483	8756	8663	8697	3755	3734	347	0	3236	3325	3744	3706	8551	8372
PU0161V2REC1	4255	4249	8504	8737	7364	7577	7379	7639	7567	7577	1673	1646	3577	3236	0	348	3068	3051	7816	7668
PU0161V2SEC1	4348	4330	8719	8961	7557	7789	7580	7814	7737	7764	1745	1742	3719	3325	348	0	3155	3168	8029	7888
PU0197V2REC1	4602	4616	9408	9771	8374	8721	8394	8779	8662	8672	3455	3424	4190	3744	3068	3155	0	354	8737	8486
PU0197V2SEC1	4550	4535	9260	9583	8231	8537	8252	8603	8494	8498	3381	3370	4166	3706	3051	3168	354	0	8621	8414
PU0210V1REC1	9878	9957	8856	9246	8357	8752	8370	8864	8679	8708	9343	9285	9965	8551	7816	8029	8737	8621	0	362
PU0210V1SEC1	9516	9588	8591	8989	8158	8550	8206	8680	8471	8514	9098	8980	9623	8372	7668	7888	8486	8414	362	0

Figure 10. PREVENT TD samples SNP distances calculated using snp-dist 0.8.2

3.4. DISCUSSION

Funding was granted for additional long read Oxford Nanopore Technologies sequencing on 30 strains to be performed sample of the *E. coli* strains. However, due to time constraints and limits on laboratory space during the ongoing COVID-19 pandemic, this was not possible.

The baseline level of ESBL does appear high, compared to other studies – further work was completed by a Military colleague in 2021. It found baseline rates of 8.4% (unpublished data, personal correspondence). Baseline samples were taken prior to deployment and any TD samples during deployment collected for analysis with a bedside multiplex PCR testing, the BioFire FilmArray (BioFire Diagnostics, Salt Lake City, UT, USA) and frozen for MDR analysis at Liverpool School of Tropical Medicine (LSTM, UK). This came after a period of limited social interaction and foreign travel as a result of the COVID-19 pandemic. These are possible factors in the reduced level compared to the PREVENT TD cohort in 2019.

It is possible, that individuals were colonised with the same strain throughout the study, but that it was identified at different time points. This is a limitation of the study from picking single colonies from each plate, rather than multiple.

There are several variables that could explain the high baseline ESBL-E level demonstrated in this cohort, unfortunately these were not recorded during the study, as it was not a primary outcome. This could include living with individuals carrying ESBL-producing *E. coli* prior to deployment; prior international travel to areas of high ESBL prevalence; environmental contamination of living spaces; and previous antibiotic use or hospital treatment.
This is a ‘known unknown’ of the study design for this secondary end-point. The pre-deployment activities and contacts of those being deployed should be recorded to try and map their ESBL-EC acquisition in future.
During most military deployments there is time for personnel to visit the local restaurants and hotels, these may also be the source of contamination. This information was unfortunately not collected for the study, but will be useful data to collect during future studies.

E. coli are able to produce carbapenemases (Ellaby *et al*. 2019). Although enrichment and selection for Carbapenemase-Producing Enterobacteriaceae (CPE) was not part of this study, no evidence of carbapenemase resistance genes was seen in these *E. coli* strains. Given the increasing CPE prevalence in the UK (Ashiru-Oredope *et al*. 2013) and worldwide (van Duin and Doi 2017), this would be worthwhile further research. Especially given that clinical treatment failure with β-lactams, including 3rd generation cephalosporins, often prompts escalation of treatment to carbapenems, such as meropenem or ertapenem (Razazi *et al*. 2012). In countries known to have high prevalence of ESBL-E, carbapenems are the first choice for treatment of sepsis, which in turn selects for CPE (Chen *et al*. 2011) (Munoz-Price *et al*. 2010) in the “arms race” between bacteria and antimicrobials.

10/35 (28.6%) of ESBL-EC strains isolated from separate individuals were of the same ST, and carried the same plasmids, virulence factors and AMR genes as the *E. coli* isolated from the UTI Chromoselect agar plate.

This suggests the ESBL *E. coli* could be the dominant strain in these individuals. This can, however, not be confidently confirmed as only a single strain was taken from each plate.

E. marmotae, is a recently described species (Liu *et al*. 2015) found originally in faecal samples of Himalayan marmots (*Marmota himalayana)* living on the Qinghai-Tibetan plateau, China. The 16S rRNA sequence showed 99.2% similarity to *E. coli* ATCC 11775. Marmots are terrestrial rodents found in northern Eurasia and North America (Steppan *et al*. 1999). The studied Himalayan marmot resides 2,800-4,000m above sea level on the Qinghai-Tibetan Plateau (Liu et al. 2019). With expansion of human activity throughout the globe, there is increasing interaction with animals that would have previously had minimal contact. There are recent case reports of clinical infections with *E. marmotae* that also comment on the similarity to *E. coli*, which may underestimate the prevalence (Sivertsen et al. 2022; Sinha et al. 2023).

Most of the sequenced *E. coli* strains had virulence genes that were associated with different *E. coli* pathoypes. However, as virulence mechanisms are more

complicated than simply the presence of genes, only a low number of individuals displayed clinical symptoms.

A minor limitation of the sequencing work was that 20/38 (52.6%) of the phenotypically ESBL-EC strains did not contain well known ESBL genes. There were explanations in the results section for some of these strains, and the presence of *blaEC* gene in all strains may go towards explaining the ability of these strains to grow on media that are selective for ESBL producers. In the time since the laboratory based research was conducted, further understanding of the *-blaEC* gene has developed, and led to the identification of variants being shown to be resistant to 3rd generation cephalosporins (Schmidt et al. 2023). .

Another possibility for phenotypic β-lactamase activity in strains that do not have traditional ESBL AMR genes, is through the action of ESBL genes from truly resistant strains, which protect neighbouring strains. This is known as "sociality" of AMR (Bottery et al. 2016).
Antibiotic resistance genes can encode cooperative resistance, which benefits both the resistant cell and the surrounding cells, whether they are truly antibiotic resistant or not (Vega and Gore 2014). An example of this mechanism would be the hydrolysis of β-lactams by secreted β-lactamases, which inactivate antibiotics in the immediate vicinity of the resistant strain. This reduction in the overall environmental antibiotic concentration will benefit both resistant and non-resistant cells (Bottery et al. 2016).
As the study protocol involved the selection and growth of a single strain per plate, this phenomenon is a possible explanation for the findings seen. Many samples had growth from various coliforms, whilst only *E. coli* were selected for sequencing. It is also possible that ESBL *Klebsiella* were seen on the agar, adjacent to sensitive *E. coli.*

CHAPTER 4: Preparation and conduct of a Randomised controlled Clinical Trial for the use of an over the counter product in the prevention of Traveller's Diarrhoea (TD.)

4.1 INTRODUCTION

Daily prophylactics are effective in reducing TD rates, but there are clinical and cost effective implications to the widespread use across a deploying force.
This was the motivation to conduct a clinical trial into the daily use of commercially available, over the counter oral prophylactics, in order to maintain gut health during deployment.

Along with US Collaborators at Infectious Disease Clinical Research Program (IDCRP) based in Washington, we are conducting "A Randomized, Double-Blind, Placebo-Controlled Trial to Evaluate Dietary Supplements to Maintain Gut Health During Deployment and Travel." (ClinicalTrials.gov NCT04605783).

This trial is known as P2 for the treatment arm and placebo:

1. Passive Immunoprophylaxis: Travelan® (Immuron Ltd, Melbourne, AUS) is a Hyperimmune Bovine Colostrum (HBC) containing specific immunoglobulin G (IgG) to 14 ETEC strains. It will be used as 400mg twice daily.

   Otto *et al* evaluated anti-ETEC Ig for 90 healthy adults in a direct challenge trial with oral ETEC 078 strain. The protective efficacy was 76.7% and 58.3% for 400mg and 200mg arms, respectively, compared to placebo (Otto *et al*. 2011).

2. Placebo control arm. The gold standard of clinical trials is placebo-controlled trials to determine true efficacy. The treatment arm will be blinded to the study participant and those that conduct the trial.

AIMS AND OBJECTIVES OF INVESTIGATIVE STUDY

Primary objective:

- To evaluate the clinical efficacy of Travelan® vs. placebo for prevention of GH disruption as measured by the combined endpoint of incidence of GH disruption (defined as 3 or more unformed stools in a 24-hour period OR 2 or more unformed stools **and** one or more associated symptoms in a 24-hour period OR antibiotic treatment for diarrhea per subject report), focusing on a 10 day window of prophylaxis during travel.

Secondary objectives:

- To evaluate the tolerability (e.g. taste, bloating, flatulence, etc.) and compliance with Travelan® during travel.
- To evaluate the risk of incident Functional Gut Disorder (FGD) and Reactive Arthiritis (ReA) in subjects in the dietary supplement arms vs. placebo.

- To evaluate the impact of the study products on the gut microbiome.
- Identify microbiome profiles associated with risk of GH disruption and abundance of antimicrobial resistance genes

Future benefit to Defence
The cost of the investigatory products is £29 for a 14-day course. The average TD episode results in 2 days loss of activity (Connor *et al*. 2013). US estimates of the value of a troop duty day range from $900 to $3,000 (£650 – £2,200) depending on the location of travel and type of mission. Thus, a product aimed at TD prophylaxis could be highly cost-effective. This could be applied as force health protection, reducing man-days lost to TD and maintaining operational effectiveness.

The study product is frequently used, well tolerated (Australian Government Department of Health 2004) and warrants investigation as prophylaxis for maintaining GH in a deploying military population. The degree of protection will be crucial in cost-effectiveness analyses and future planning. This represents a paradigm shift from treatment to prevention, in a potentially cost-effective and easily distributed manner, that can be scaled up to whole force health protection.

4.2. STUDY PROCEDURES

Study personnel will dispense the study product (labelled with the appropriate randomisation number) in sachets ('AM dose' and 'PM dose') to the study participant. Subjects will be randomised, equally to the 3 study arms (all subjects will take 1 sachet twice a day, for the first 10 days of travel.) This will include 434 in each treatment groups, totalling 868 study participants.

Study participants will also be asked to provide a stool and serum samples at the beginning and the end of the study. They will keep a stool and symptom diary, which will be collected at the end of study. These will be analysed for the clinical outcomes of the study.
Individuals will then also be emailed follow up surveys at 3 and 6 months to monitor for FGD and ReA. The FGD assessment will use ROME IV criteria (Lacy et al. 2016).

Data will also be electronically captured from database sources. Study data will be collected at the study sites and maintained in a standardised format. It will be completed on an ongoing basis using the electronic data capture (EDC) platform Research Electronic Data Capture (REDCap). The data will be uploaded to the IDCRP Data Co-ordination Centre (DCC) for inclusion in the study database.
Stool and serum samples from the study will be frozen at site laboratories at -80°C.

STATISTICAL CONSIDERATIONS

A target sample size is 868 to be divided between the 3 locations. Power calculations are based on the assumption of 35% maintenance of GH/ TD prevention. There is an anticipated 25% placebo attack rate, with a 25% loss to follow up. In order to achieve an 80% study power, 330 study participants are needed for each of the 4 study arms. The UK trial site.

The study will not compare the study products versus placebo in an intention to treat (ITT) analysis. This analysis will include all subjects who are randomly assigned to the study product and received at least one dose prior to travel.

The randomisation schedule and codes will be created using SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA) by an IDCRP programmer and provided to the research team at each enrollment site. Randomisation assignments will be made in permuted blocks by site using a 1:1:1:1 randomisation. The block size will maximise ease of use, maintenance of blinding, and balance of prognostic factors in study arms.

ELIGIBILITY CRITERIA

Inclusion Criteria

1. 18-70 years old, able to read and speak English fluently and provide informed consent.
2. Travel or deployment with minimum of 10 consecutive days at intermediate to high risk for GH disruption destination.
3. Ability to complete a follow-up visit within 30 days of the end of taking study product.
4. Departure date < 7 days from the date of enrollment.
5. Willing to comply with study procedures.

Exclusion Criteria

1. Subject-reported history of any known FGD (including Irritable Bowel Syndrome) or chronic gastrointestinal disease (e.g. Inflammatory Bowel Disease) which would preclude assessment of study outcomes.
2. Antibiotic use within 7 days prior to enrolment (except for malaria prophylaxis including doxycycline, chloroquine, atovaquone/proguanil, mefloquine and primaquine).
3. Experiencing diarrhoeal illness (defined as 3 or more loose/liquid stools in a 24-hour period) within 3 days prior to enrollment.
4. Planned use of any investigational or non-registered drug, antibiotic or other probiotics or prebiotics (outside of the study product) during the study period. This does not include consumption of yogurt products.
5. Intended use of a GH disruption prophylactic (e.g. Pepto-Bismol, rifaximin) during the study period.
6. Any planned medication usage during the study period that is deemed to interfere with gastrointestinal function, including but not limited to anti-diarrhoeal medication and prokinetics.
7. Any confirmed or suspected cancer, or use of immunosuppressant medication (topical steroids are permitted) in the last 6 months which in the opinion of the investigator would impair interpretation of the study data.

STUDY VISITS

The study has been designed in collaboration with the Deployment and Travel-Related Infections Research Area of the Infectious Disease Clinical Research Group (IDCRP), Uniformed Services University, Bethesda, MD, USA.

P2 will recruit UK Military personnel, US Military personnel and UK Civilian Travellers (via the Liverpool School of Tropical Medicine, LSTM). A total of 868 study participants are to be recruited, divided equally between the 2 study arms. There will be no interaction between investigators and study participants from different sites.

Separate regulatory authorisation is being sought for the US study sites, thus the remainder of the study design, methods and data analysis will concentrate on UK specific features, where possible.

UK study participants will be identified as those deploying to areas of high risk for GH disruption.

Interventional Groups & Study Product Administration

Participants will be consented, enrolled and randomised to one of 4 treatment arms:

- 434 participants - Travelan® 600 mg twice daily with meals
- 434 participants - Placebo (maltodextrin) twice daily with meals

Study participants will be enrolled prior to departure overseas and will begin taking the study product 3 days prior to arrival at destination, continuing for 10 days during travel. They will be provided with a total of 13 days of study product.

Participants will be asked to take 1 sachet twice a day with meals. They will be instructed to keep the study product at ambient temperature and in a dry location away from children. Unused doses of the study product will be returned by the participant to study personnel and destroyed by study personnel at the completion of the study.

Investigational Study Product

Study product will be repackaged into blinded sachets by a subcontracted pharmacy in the US by Southwest Research Institute (SwRI) pharmaceuticals, San Antonio, Texas. They will be indistinguishable. An inert pigment will be added along with vanilla flavouring, in order to make the powders an identical colour and taste. Preparation of study regimens will be based on a block randomisation. The box of sachets will be labelled with the study identification number and the appropriate randomisation number as per the above schedule and stored in the clinical research coordinators office.

Randomisation & blinding procedures

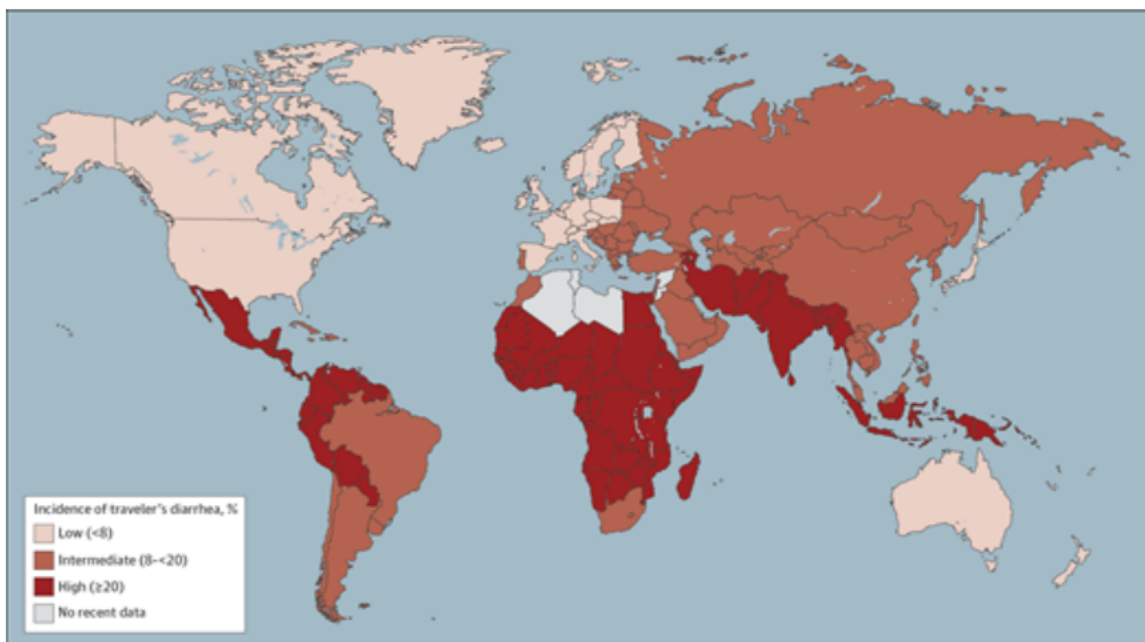
The randomisation schedule and codes will be created using SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA) by an IDCRP programmer and provided to the research team at each enrollment site. Randomisation assignments will be made in permuted blocks by site using a 1:1:1:1 randomisation. The block size

will maximise ease of use, maintenance of blinding, and balance of prognostic factors in study arms.

Study visits and procedures.

In order to accommodate the variability in deployment schedules and periods of GH disruption risk, enrolment will occur mainly in the UK firm base, prior to deployment. This will allow us to recruit those deploying to the highest GH risk areas, who may be part of small teams, with limited seats on the transport and very limited laboratory facilities in their country of destination.

Regions of high GH disruption risk are identified with reference to published data on TD risk (Steffen et al. 2015).



Countries with ≥ 20% risk of TD (based on Travelers Diarrhea: A Clinical Review. JAMA 2015; 313(1): 71-80)

Central America	South America	Africa		Asia
Belize	Colombia	Algeria	Burundi	Armenia
Costa Rica	Bolivia	Egypt	Comoros	Azerbaijan
El Salvador	Ecuador	Libya	Djibouti	Palestine (Occupied Palestinian Territory)
Guatemala	French Guiana	South Sudan	Eritrea	Syria (Syrian Arab Republic)
Honduras		Sudan	Ethiopia	Afghanistan
Mexico	Guyana	Morocco	Kenya	Bangladesh
Nicaragua	Paraguay	Western Sahara	Madagascar	Bhutan

Panama	Peru	Botswana	Malawi	India
	Suriname	Lesotho	Mauritius	Iran (Islamic Republic of)
	Venezuela	Namibia	Sao Tome and Principe	Maldives
		Swaziland	Benin	Nepal
		Angola	Burkina Faso	Pakistan
		Cameroon	Cape Verde	Sri Lanka
		Central African Republic	Cote d'Ivoire	Christmas Island
		Chad	Gambia	Indonesia
		Congo	Ghana	Myanmar
		Dem Rep of Congo	Guinea	Timor-Leste (East Timor)
		Equatorial Guinea	Mayotte	Norfolk Island
		Gabon	Mozambique	Fiji
		Guinea-Bissau	Réunion	New Caledonia
		Liberia	Rwanda	Papua New Guinea
		Mali	Seychelles	Solomon Islands
		Mauritania	Somalia	Vanuatu
		Niger	Uganda	Guam
		Nigeria	Tanzania	
		Saint Helena	Zambia	
		Senegal	Zimbabwe	
		Sierra Leone	Tunisia	
		Togo		

Enrolment visit:

- Potential participants will be identified after attending a briefing and being given 24 hours to consider prior to providing informed consent. This is covered in more detail in Sections 15 and 16.
- Enrolled participants will complete a survey that collects demographics, itinerary and a baseline assessment for FGD and ReA.
- A blood specimen will be collected (up to 17 mL), and participants will be asked to submit a fresh stool specimen prior to travel – this will act as a baseline/ control sample. Instructions how to use the stool collection kit is provided in the Patient Information Sheet (PIS).
- Participants will be randomised to receive one of three active agents or masked placebo in a 1:1:1:1 manner, as described above. All participants will take 1 sachet, twice daily.

- Participants will be issued a 13-day course of study product (i.e. starting the regimen 3 days prior to arrival at destination and continuing for 10 days in the study location.)
- Participants will be provided with a paper travel diary to capture episodes of GH disruption, use of antibiotics and anti-diarrhoeals for the treatment of diarrhoea. They will also be provided with an adherence log for the study product and use of concomitant medications.
- Study participants will be asked for permission to use their stored stool and serum for future analysis. They will also be asked to allow us to review their medical notes looking for evidence of FGD, ReA and associated conditions, over the next 5 years. This will act as an additional safety feature to assess for the development of long-term health problems and is included in the consent forms. This has received Defence Medical Service (DMS) Caldicott Guardian approval.

During deployment:

- For every GH disruption episode experienced, the participants will record the date, time, symptoms experienced, the effect on activity level and consumption of products for GH disruption in the travel diary. In cases where the duration of travel exceeds 10 days, participants will continue to document any GH disruption episode after completion of study product. The diary will also capture the start and stop dates of study product and missed doses of study product.
- Study participants will be asked to self-collect a stool smear on a stool card within the first 5 days of being in the GH disruption risk destination. The process of this will be explained in the PIS, including an easy-to-follow diagram. Sterile gloves and the required kit will be provided. This process will be discussed in the recruitment study visit.
- Study participants may be contacted by investigators travel in order to foster adherence.
- Study participants who experience episodes of GH disruption will be asked to self-collect a stool smear on a stool card for 2 consecutive days (one per day) of the episode. Stool cards will be stored in a sealed bag and shipped to the study site or brought back after return for the in-person visit.
- Study product will be continued for the prescribed duration regardless of whether a participant experiences GH disruption or takes self-treatment.
- Study participants in BATUK, Kenya (or elsewhere if laboratory facilities allow) will be asked to provide a fresh stool, which will be frozen to -80°C, for transport back to the UK via an established cold chain.

Follow-up visit:

- Follow-up visits should occur within 30 days of completion of taking the study product. They can happen from the day after completion of study products. This may occur in the country of deployment, or following return from deployment, depending on the length of deployment and the availability of study investigators. This 30 day period can be extended for deployment

reasons.
Final visits can be made remotely, if secure IT infrastructure allows, and if a face-to-face final visit is impractical. This is very possible currently, especially due to COVID restrictions. The travel diary data will be entered on to REDCap by the study investigator during the virtual follow-up visit. We will use pre-paid envelopes to collate the source data & stool cards.
This virtual follow-up will be used to maximise collection of primary outcome data, whilst taking into consideration the possible restrictions due to the ongoing COVID-19 crisis.

- Participants will be contacted by study staff during travel or immediately after return to confirm or schedule their follow up appointment.
- Unused products (study product, stool cards) will be returned and destruction following UK legislation assured by study investigators.
  The study team will collect the travel diary and stool cards.
- A serum specimen (up to 17mL) and stool specimen will be collected. This will not be possible with virtual follow-up visits.
- Participants will complete an online survey at 3 and 6 months after return to evaluate for symptoms of FGD and ReA.
- A note will be included on UK study participants medical records (DMICP) including the dates of their study involvement and giving contact details of the Principal Investigator (PI) and Clinical Research Co-ordinator (CRC). This will allow study participants medical teams to contact the study investigators if issues arise once the participant has completed their involvement in the study.

An easily understandable infographic is included in the study PIS.

A participant may voluntarily end participation in the study at any time. If a participant withdraws, study staff will make a reasonable effort to determine the reason for the participant's withdrawal from the study. Telephone calls and/or e-mail correspondence will be considered reasonable effort. All data and specimens collected up to the time of withdrawal will be reported and retained.

Data Collection

Study data will be collected at the study sites and maintained in a standardised format. Data collection will be completed on an ongoing basis during the study using the electronic data capture (EDC) platform, Research Electronic Data Capture (REDCap). Source document worksheets will be used by study investigators prior to entering data into REDCap. The electronic data will be uploaded regularly by US IDCRP Data Coordination Center (DCC) for inclusion in the study database.

The methods and instructions for data collection will be distributed to the site. Any changes made to data will have an associated audit trail. The study investigator is responsible for assuring that the data collected are complete, accurate, and recorded in a timely manner. Source documentation (the point of initial recording of information) should support the data collected on the study form. Data will be collected directly from participants during study visits and/or telephone calls and will be abstracted from participants' diaries.

Data Management

Participants will be identified on study forms, serum, stool card, and stool specimens by a unique participant identification number. Other than name and email address (to be used for email distribution) no personal identifiers will be transferred to the data management or data analysis study personnel. Data collected through this protocol will be stripped of participant identifiers for entry into the data management system and be maintained with highly limited access. The code linking participant ID to personal identifiers will be kept on a password protected file on a computer that requires a password to access. Access will be restricted to study personnel requiring the information for study procedures. All essential documentation will be retained by the institution for 10 years, as required by MODREC. No study document should be destroyed without prior written agreement between the IDCRP and the Site PI. All samples collected under this protocol may be retained indefinitely.

The site will follow all confidentiality protections and requirements from the Data Protection Act (DPA) 2018 and General Data Protection Regulation (GDPR), De-identified research data will be collected by and sent to the US IDCRP Data Coordination Center (DCC.)

This will include study participants email addresses, which will also be stored on the secure server at US IDCRP DCC, in order to send out the Amazon voucher codes and follow-up surveys at 3 and 6 months. Once the email address has been used, it will be destroyed. This automated process is programmed into the REDCap program and a confirmatory email will be sent to the site PI.

A technique in a previous MODREC authorised protocol (770/MODREC/16) involved sending the follow-up emails by a member of the study team. This automated technique would prevent human error causing the email to be typed incorrectly or email addresses being shared by inadvertent cc'ing, thus breaching confidentiality.

Participant data will be protected following industry standards and best practices. This includes the highest level of encryption, Multi Factor Authentication, restricted access to the data centre, Role Based Access provisioning, and a robust auditing system.

Study research data may only be entered into or viewed on the data management system by users who are given specific role-based permissions to do so. Access to participant's personal data will be highly limited. Data are stored on a secure server at IDCRP in Henry M. Jackson Foundation (HJF) for the Advancement of Military Medicine. Access to the server is only granted to authorised administrators.

Human Biological Specimens/ Tissue (HBS)

Methods of specimen collection, handling, and processing as well as details of specimen storage, maintenance, and quality assurance/ quality control (QA/ QC) are described in the Laboratory Manual. Stool and serum samples from the study will be housed in the University of Birmingham, under the supervision of the site PI. This will be in a dedicated -80°C freezer with temperature monitoring. Pro-curo software

(Horsham, UK) will be used to register and track stool samples, ensuring Human Tissue Act (HTA) 2004 compliance. Some will then be transferred to the United States Military HIV Research Program Specimen Processing Laboratory (SPL) and Naval Medical Center Portsmouth, USA (NMCP). Those collected overseas, where facilities exist, will be stored appropriately and transferred back to the UK via an established cold chain.

Managing Data and/ or Human Biological Specimens for future research
Data collected through this protocol will be stripped of participant identifiers for entry into the data management system and be maintained with highly limited access. Data and samples released for research will be labeled by participant ID only; data will be shared without any identifiers or links to individuals. Additionally, participants may decide at any point not to have their samples and data stored any longer or used for future research; this decision will not affect the participant's participation in this protocol. In this case, the principal investigator (PI) will assure the destruction of all known remaining samples and report the destruction to both the participant and to all participating RECs. Residual samples (samples from participants who have consented to storage of their samples) will be preserved for future analysis indefinitely, acknowledging that any analysis outside of the scope of this protocol will be requested through each REC.

Data Analysis
Primary Endpoint (Efficacy):

The primary objective is to evaluate the clinical efficacy of Travelan® versus placebo for GH maintenance.

- Primary endpoint data will be obtained from review of travel diaries completed by study participants during the 10-day travel period. Participants will collect the number of episodes of GH disruption in one of the three 8-hour time-block periods, which will also include subsidiary symptoms during GH disruption episode and any usage of antibiotics and anti-diarrhoeals.
- The primary outcome is to assess the maintenance of GH compared between the active agent vs. placebo utilising the GH disruption proportion in a 10-day period. GH disruption is defined as 3 or more unformed stools in a 24-hour period OR 2 or more unformed stools and one or more associated symptoms (nausea, vomiting, abdominal pain, fever, bloody stool) in a 24-hour period OR treatment of diarrhoea with antibiotics per participant report, focusing on a 10-day window during travel.
- Participants who terminate early to due to the onset of GH disruption without meeting the formal definition of GH disruption will be treated as occurrences of treated GH disruption if they were treated with rescue antibiotic medication; otherwise they will be censored at the time of their last dose of the active agent.

The incidence of GH disruption will be compared utilising the number of GH disruption events and compared across study arms. Participants baseline

characteristics and summary follow-up findings will be compared using analysis of variance and other general linear model applications methods, including Mann-Whitney U-statistic and Kruskal-Wallis tests and chi-square or Fisher's exact tests as deemed appropriate. Baseline demographics frequencies will be collected from pre-deployment/ travel survey and itinerary forms.
Sub-group analysis will also be performed (by region) if primary outcome is not achieved. This will allow for better translation of the research to the deployed service person.

Clinical summaries of reported tolerability will be presented including the frequencies and duration and association with non-compliance. Descriptive statistics will be used to measure difference in the distribution of enteropathogens associated with GH disruption and to evaluate the risk of incident FGD and ReA in participants in the dietary supplement arms vs. placebo. The association between GH disruption and the odds of meeting FGD (as defined by the ROME IV criteria) or ReA definitions will be used to estimate the risk development of the outcome for each study arm.

Exploratory Analysis: Descriptive statistics will be used to evaluate exploratory endpoints:

- Severity (by frequency) and duration of GH disruption episode
- Duration of GH disruption episode (i.e. time to complete resolution of GH episode), impact on daily activities (i.e. complete and partial incapacitation) and health care utilisation during travel
- Clinical efficacy and tolerability for participants with a duration of taking study product of greater than 10 days
- Clinical efficacy as measured by the time to first unformed stool (TFUS) from baseline. Kaplan-Meyer methods will be utilized to measure median TFUS
- Incidence of delayed onset of GH disruption in participants with travel durations longer than the period of taking the study product
- The efficacy of Travelan® in various geographic regions using enteropathogen data from stool card PCR testing

Incidence of GH disruption will be used to evaluate the efficacy for the study product. Relative risk of GH disruption based on travel destination will also be assessed. Other predictor variables including age, gender and rank using logistic regression models to estimate the odds ratios and assess for confounding between the primary predictor variable (presence or absence of GH disruption during deployment) and other predictor variables.

The following exploratory endpoints will be conducted based on the availability of additional funding:

- To evaluate the impact of Travelan® use vs. placebo on the gut microbiome and virome during deployment and travel
- Identify microbiome and / or virome profiles associated with risk of GH disruption and abundance of antimicrobial resistance genes

- Obtain parameter estimates to inform a cost-effectiveness model of dietary supplement use to maintain GH
- Evaluate the impact of compliance with dietary supplement use on maintaining microbiome density and diversity
- Describe the seroprevalence of biomarkers associated with chronic sequelae of GH disruption in participants who develop FBD and asymptomatic controls

Characterise the humoral immune responses in participants with GH disruption and enteropathogens detected by the TaqMan PCR assay on stool smears. Use serum proteomic profiling to describe differences in the host immune response to GH disruption among participants on dietary supplements or placebo.

CONSENT

In the UK, after the explanation about the study is completed, individuals will be given the opportunity to ask for additional information they desire about the study. Individuals will read the Subject Information Sheet and will sign the informed consent. UK Ministry of Defence Research Ethics Committees (2050/MODREC/21) cleared policy will be followed at UK cohort recruitment sites. Subjects will be informed that their participation in the study is voluntary and persons who elect not to participate will be treated in the same manner as persons who elect to participate in the study. The consent form/signature page will be signed, copies will be given to the subject, and the originals will be maintained at the site.

PARTICIPANT INFORMATION SHEET

Study title: *A Randomised, Double-Blind, Placebo-Controlled Trial to Evaluate Dietary Supplements to Maintain Gut Health During Deployment and Travel (P4)*

MODREC Application No: 2050/MODREC/21

Invitation to take part:
You may be eligible to take part in this research study. This form gives you important information about the study.

Please take time to carefully review this information. You should talk to the researchers about the study and ask them any questions you have. You may also wish to talk to others (for example, your friends, family, or Medical Officer) about your participation. You do not have to take part in this study. Participation is voluntary. You may also leave the research study at any time without any impact on your future care our healthcare.

What is the purpose of the research?

Maintaining gut health (GH) is a common problem during military deployment and overseas travel and can lead to other health problems. Disruption of gut health is commonly caused by eating food or drinking water that is contaminated with germs (bacteria, viruses, or parasites). Even being careful with what you eat and strict hand hygiene, many people still develop disruption of the gut, especially if circumstances make it hard to follow the guidance.

Dietary supplements offer a potentially safe and effective method of maintaining gut health by improving the body’s defence against gut pathogens. This may also reduce the need to use antibiotics.

The purpose of the study is to evaluate how well three dietary supplements work in maintaining gut health. The study will also look at the balance of good and bad bacteria in stool and its association with Travellers’ Diarrhoea (TD).

The dietary supplements being studied is Travelan®. Travelan® is available over the counter in Australia and Canada.

- Travelan® is comprised of proteins that stop the toxins from Enterotoxigenic *E. coli* - a harmful bacteria that causes most cases of TD.

There is evidence to show these dietary supplements reduce gastrointestinal illnesses and may help maintaining gut health.

Please tell our research staff if you have been prescribed or intend to take other medications whilst deployed, or if you are participating in other research studies.

Who is doing this research?
We are the Academic Department of Military Medicine (ADMM) based at Royal Centre for Defence Medicine (RCDM). We are collaborating with the US Infectious Disease Clinical Research Program (IDCRP). Study participants are being recruited from both the UK and USA.

The research has been paid for by our US collaborators. They are co-ordinating the study and are responsible for analysing study data. There will be no interaction between UK study participants and US study investigators, or vice versa.

There are no drug companies involved in the research.

Why have I been invited to take part?
We are recruiting UK Military personnel, prior to their deployment overseas to an area of high risk for gut health disruption. You are being asked to take part in this research study because you are planning to travel to areas outside of the UK with an expected risk of this.

There will be 868 people taking part in this study overall, with 300 study participants from the UK Military and the remainder from our US collaborators.

Before you can take part in this study, you will need to provide some information so that the Investigator can confirm that you qualify for the study. This is called the "Screening Process". You will be asked to provide information about your upcoming trip, current use of medications, and history of gastrointestinal diseases/disorders. You are not able to participate in the research study if you have a history of certain chronic bowel conditions such as irritable bowel syndrome.

Do I have to take part?

No. The decision to take part in this research study is completely voluntary, which means you do not have to take part if you do not want to. You may also leave the research study at any time. If you choose not to take part in this research study or if you leave the study before it is finished, there will be no penalty or loss of benefits to which you are otherwise entitled.

You will be informed if significant new findings develop during this research study that may relate to your decision to continue participation.

What will I be asked to do?
During the study, you will have two visits with study personnel. The first, prior to departure and another following your return. Each visit will take up to 45 minutes to complete. Additional surveys will be sent for to you to complete online at 3- and 6-months after returning from deployment. The total expected duration of the research study enrolment will be 2 years, however, your participation in the research study will last for up to 6 months after return from travel.

The main things you will be asked to do are taking a dietary supplement twice a day for 13-23 days, providing 2 blood samples and 2 stool samples, and completing surveys before, during, and after travel. You will be asked to collect a stool smear (in private) on a filter paper card during the first few days of travel and during any episodes of diarrhoea.

Since we do not know which dietary supplement is better for maintaining gut health than taking nothing, we need to compare each of them against a placebo. A placebo is an inactive, harmless substance, like a sugar pill, that looks like the research study medication but contains no medication.

You will be randomly assigned to one of 2 groups. Randomisation is a process like flipping a coin and means you will have an equal chance of being assigned to either group:

1) Travelan®
2) Placebo

Whichever group you are randomised to join, you will take 1 sachet (packet) of the product two times each day with the two biggest meals of the day, ideally once in the morning and once in the evening. If you do not eat two meals each day, you may take it with snacks or without food. You will start taking the product 3 days before you arrive in your overseas destination and then continue taking it for 10 days during travel.

This is a double-blind research study, which means that neither you, nor the research team will know whether you are receiving the research study product or a placebo. In the event of an emergency, there is a way to find out which one you are receiving.

<u>Your Participation in This Study Will Involve the Following Events:</u>
Prior to Travel

- Collection of contact information. This information may be used by study personnel to contact you during your participation in the study.
- You will be asked to sign a consent form.
- Complete surveys that will ask questions about personal information (e.g. age, gender), your trip itinerary, and assess if you have symptoms of functional bowel disorders, such irritable bowel syndrome (IBS) and arthritis. The surveys will take about 20-30 minutes to complete.

- You will collect a stool (faeces) sample (in private) and provide it to study personnel, prior to your departure. Please see the attached instructions for using the stool collection kit.
- A blood sample will be collected by a trained member of the team (approx. 1 tablespoon - 17 ml).
- You will start taking the study product 2 times each day, starting 3 days prior to arrival at travel destination.

During Travel

- You will continue taking the study product 2 times each day for 10 days during travel.
- You will collect a stool smear on a card provided to you within the first 5 days of arrival at the travel destination.
- If you develop diarrhoea, please collect a stool smear sample on a card each day during that diarrhoeal episode (up to a max of 2.)
- You will complete a travel diary. This will collect information on the dates you start and stop the study product, the number of times you miss the study product, and any symptoms you experience during travel.

After stopping taking the product

- You will meet with study personnel and return the stool cards and completed travel diary. You will complete a brief survey regarding behaviours related to gut health disruption, tolerability of study product, and ability to take study products with food.
  This may be completed virtually, if the COVID restrictions require it.
- Another blood specimen will be collected (approximately 1 tablespoon - 17 ml).
- You will collect a 2nd stool (faeces) sample and provide it to study personnel.
- This may happen in the UK or in the host country, depending on where you have been deployed to.

Some travel-associated illnesses can persist or show up after an initial diarrhoeal illness. An additional procedure for this research study consists of a set of follow-up surveys. These will assess the existence of functional bowel disorders, such as Irritable Bowel Syndrome (IBS) and Reactive Arthritis (ReA.)

If you agree to participate in the study, you will be asked to complete the surveys 3- and 6-months after you return from travel. You will be asked to take the surveys online. A link will be emailed to you on the address provided during study recruitment. The survey will take approximately 15 minutes to complete. Study personnel will not use your e-mail address or phone number for any purpose other than this study.

An entry will be made on your Military Medical Records, explaining that you were part of this study and giving contact details of the study investigators, should your Medical Officer need to contact them about it. This will allow the research team to follow you up, looking for travel or deployment related illnesses for up to 5 years.

With your permission, your stool (faeces) and serum (part of blood) samples will be kept for future research. This would likely be to investigate other aspects of infections that may affect deployed Military personnel. Your name and any information that could link to your identity to the samples will be removed.

Outline of participant journey

Pre-Deployment	Complete enrolment paperwork.	Recruitment and randomisation of study product 5 – 7 days prior to travel.	Provide blood and fresh stool sample prior to travel. Receive compensation (£30).	Start study product 3 days prior to travel.	Begin completing the Travel Diary.
During Deployment	Continue taking the study product (1 sachet, 2 times a day with meals) for 10 days.	Collect a stool card within the first 5 days of arrival at travel destination.	If you develop diarrhea, collect a stool card for 2 consecutive days during the diarrheal episode.	Continue Travel Diary, recording use of study product, illnesses experienced, and use of treatment for illnesses.	
Post Deployment	Within 4 weeks of return appointment with study team.	Return stool cards and completed Travel Diary.	Provide blood and fresh stool sample post travel. Receive compensation (£30).		
3 and 6 months	Complete web survey at 3 months. Receive compensation after completion (£15)	Complete web survey at 3 months. Receive compensation after completion (£15)			

Are there any direct benefits to me of taking part?
Yes, possibly. There is the potential that a study product may help maintain gut health during and immediately following your overseas travel. However, there is no guarantee that you will benefit from being in this research.

There may be other medications for maintaining gut health, however, none of them are currently approved for this purpose. The products involved in this research study may also be available over the counter without taking part in this study. You may talk with your Medical Officer about these options.

Choosing not to take part in this research study is also an option. Your participation is entirely voluntary. There may be other research studies involving prevention strategies that could be helpful in maintaining gut health.

What are the possible disadvantages (or risks) of taking part?
If you choose to take part in this study, there is a risk of:

- Allergic reactions – This has not been reported in previous studies using these products but could be a rare event occurring in less than 1 in 100 individuals.
- Infection at the site where the blood is drawn (less than 1 in 100 individuals).
- Bruising or pain at the site where blood is drawn (1 or 2 in 100 individuals)
- Diarrhoea, bloating, flatulence, rash, nausea, vomiting or stomach-ache may occur in 1 to 10 out of 100 individuals. In previous studies, these side effects were no more common than those who took the placebo compared to those who took the study drugs.

Although efforts are made to protect your research study records, there is always a small risk that someone could get access to the personal information in your medical records or other information researchers have stored about you.

You should understand that there is the possibility that the medications may not be effective in the prevention of diarrhoea. There may also be other risks of taking part in this study that we do not yet know about.

Can I withdraw from the research and what will happen if I withdraw?
Yes. Should you wish to withdraw, you must contact the Principal Investigator and/ or study personnel at the site where you enrolled. If you decide to no longer participate in this research study, the researcher will keep your data and specimens that were collected during this research study and collect any unused research products from you.

The Principal Investigator of this research study may terminate your participation at any time if he determines this to be in your best interest, if you are unable to comply with the procedures required, or if you no longer meet eligibility criteria.

The sponsor of this research study may terminate the research study and/ or your participation in this research study for safety reasons.

Will I receive any expenses or payments?
Yes, you will receive a total of £90 in Amazon vouchers. This includes a £30 Amazon voucher for the enrolment blood draw and another £30 Amazon voucher for the post-travel blood draw. You will receive £15 Amazon voucher for completion of each of the 3- and 6-month surveys. Codes will be provided by email following the study visits. The voucher codes for the follow up surveys will be emailed following completion of the surveys.

We need to inform you that as with any money you earn, it is your responsibility to pay Income Tax and National Insurance. If you are unsure about how to do this, please ask your units' Regimental Administration Officer (RAO) who will be able to tell you what to do or give you advice on how to find out.

There are no costs to you for taking part in this research study. The study investigators will travel to you for the study visits and everything you require to take part in the study will be provided.

Will my taking part or not taking part affect my career?
No. Your decision will not affect your Military career or your Medical care. If you decide to take part in this research study, you will be asked to sign the consent form. Before you sign the consent form, make sure you understand what the research study involves, including the benefits and possible risks. Please ask any questions to the study investigator.

Who do I contact if I have any questions?

Name: Dr. Tom Troth

Address: Academic Department of Military Medicine,
Research and Clinical Innovation (RCI),
RCDM, BIRMINGHAM, B15 2SQ

Tel No:

E-mail:

Who do I contact if I have a complaint?

Name: Alexandra Young QARANC
Troop Sergeant, 1 Armoured Medical Regiment

Address: Bhurtpore Barracks
TIDWORTH
SP9 7AS

Tel No:

E-mail:

What happens if I suffer any harm?
If you suffer any harm as a direct result of taking part in this study, you can apply for compensation under the MOD's No-Fault Compensation Scheme.

What will happen to any samples I give?
During this research study, you could be asked to provide blood and stool samples. While this study is on-going, your samples will be handled in accordance with this study's protocol.

The information and specimens that we obtain from you for this study might be used for future studies. We will anonymise the data and samples, by removing anything that might identify you. If we do, that information and specimens may then be used for future research studies or given to another investigator without additional permission from you.
If we want to share any information that may identify you, a review board will decide whether we need to get additional permission from you.

Future Use of Data

The investigator has requested to save selected data collected from your participation in this research study for possible use in future research. Your participant ID, which is assigned at enrolment, and is considered an identifier, may be used or shared for future research. You have a number of options with regard to this request. If the stored data has an identifying link you can request to be contacted and sign a separate consent form to allow the use or available of this data in another study. You may also choose either to not allow any further use of your data, allow use of only de-identified data, or give consent now for the use of your identifiable data to be used in future studies. This future research may be in the same area as the original study, or it may be for a different kind of study. You will be provided choices on the consent form to allow or deny use in future research studies. This is point 5 on the consent form.

Any future research using your retained data will require a research protocol for the proposed study approved by MODREC (the MOD ethics review board responsible for safeguarding and protecting research participants.) The data protections for privacy and confidentiality described in this consent form will apply to any future use of your stored data.

Future Use of Biologic Specimens

The investigators in this study are asking for your permission to store your samples for future use in other research studies. The specifics of these future research studies are unknown at this time, but these studies will frequently be in the area of diarrheal disease and evaluating changes in the composition of gut microorganisms due to travel or TD.

Your samples would be stored with the following information: study ID (your unique participant ID issued at enrolment) and the date the sample was collected. This is considered identifying information and can potentially be traced back to you as the donor.

The storage (bank) area for blood specimens is maintained at Specimen Processing Laboratory, Military HIV Research Program, 1600 East Gude Drive, Rockville, MD 20850. The storage bank Manager is responsible for the storage bank. The Manager's phone number is: (301) 251-3032 or (301) 251-3034. While this study is on-going, your stool samples will be handled in accordance with this study's protocol and applicable regulations.

Future research investigators requesting portions of your samples must have the approval of the Principal Investigator and also must have a research protocol for their newly proposed research study approved by a Research Ethics Committee (REC). It is possible these other researchers will request approval to contact you in the future.

Your samples could be stored indefinitely at the bank, or until none is left to use. Generally, you will not be provided with the results of the future studies using your samples from this bank. This is typically the case because the research results at that early point will not have a clear meaning for or direct clinical benefit to you.

You may request that your specimen be withdrawn from the bank at any time if you decide you no longer want to participate. This can be done by notifying the bank Manager listed above.

Due to the nature of the type of data that will be collected and specimen testing that will be completed, we do not anticipate any incidental or unexpected findings that would impact participants.

Will my records be kept confidential?

Yes. Confidentiality is very important to us. Please read this section particularly carefully and ask any questions you may have. We are required under the Data Protection Act (DPA) 2018 and the General Data Protection Regulation (GDPR) to tell you how your personal data may be used. Personal Data means information about you, that can identify you to someone else. This will include your name and other information collected as part of this research study.

Any personally identifiable information obtained during this study will remain confidential in your study and/ or medical file. Only information that has been made anonymous (that is, cannot be identified in any way as having been given by you) will be kept separate from these files. This anonymous information will be put together with all the other participants' information and used to allow us to work out the results of the research study. All information will be participant to the current conditions of the DPA 2018.

To ensure we run the research study to the highest standards, we have independent monitors who, from time to time, inspect the work we are doing and some of the information we are collecting. These people may see some of your personal information (specifically the consent form you sign). This is to ensure we have done things properly and for no other purpose.

Your Medical Officer will be aware be of your participation in this research study and will be made aware of any results from tests performed which may need follow up. You should be aware that by signing consent to take part in the research study, you are agreeing to this.

Anonymous research data will be entered onto the online Research Electronic Data Capture (REDCap) which is a secure, web-based application and database designed to support data capture for research studies. It includes the highest level of encryption, Multi Factor Authentication, restricted access to the data centre, Role Based Access provisioning, and a robust auditing system. This will be stored at the Data Co-ordination Centre (DCC) at the IDCRP, USA. Access to the data is only granted to the study team. The sites will follow all confidentiality protections and requirements from the Data Protection Act 2018 and General Data Protection Regulation (GDPR).

Who has reviewed the study?
This study has been reviewed and given favourable opinion by the Ministry of Defence Research Ethics Committee (MODREC)

Disclosure of financial interests and other personal arrangements
There are no financial interests or other personal arrangements to disclose

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Compliance with the Declaration of Helsinki
This study will be conducted in accordance with the principles defined in the Declaration of Helsinki [1] as adopted at the 64th WMA General Assembly at Fortaleza, Brazil in October 2013.

[1] World Medical Association Declaration of Helsinki [revised October 2013]. Recommendations Guiding Medical Doctors in Biomedical Research Involving Human Subjects. 64th WMA General Assembly, Fortaleza (Brazil).

CONSENT FORM

Title of Study: *A Randomized, Double-Blind, Placebo-Controlled Trial to Evaluate Dietary Supplements to Maintain Gut Health During Deployment and Travel (P2)*

MODREC Reference: 2050/MODREC/21

Please Initial or Tick Boxes

1. **I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to ask questions and have had these answered satisfactorily.** ☐
2. **My participation is voluntary and I am free to withdraw at any time, and ask for my samples to be removed from the study, without giving any reason, without my medical care or legal rights being affected.** ☐
3. **I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as confidential and handled in accordance with the provisions of the Data Protection Act 2018.** ☐
4. **I consent to the storage of my stool and serum specimens.** ☐
5. **(Optional)**
I consent for the use of my stool and serum samples for potential future research studies that would likely investigate other aspects of infections that may affect deployed military personnel. My name and any information that could link my identity to the samples will be removed. ☐
6. **I consent to the publication of my anonymised data combined with others to provide study results.** ☐
7. **I agree to take part in the above study.** ☐
8. **(Optional)**
I agree to take part in the study procedures of completing follow-up surveys to assess for functional gut disorders and reactive arthritis. I understand this will require sharing me email address with the US IDCRP, where it will be stored on a secure server, until it is deleted after the 2nd (6-month) follow up survey. ☐

9. **An appropriate entry will be made in my service medical documents on completion of the study. This will allow my Medical Officer to contact the study team if required. It will also allow the research team to identify and access my medical records in the future (for up to 5 years) in order to gather information about the development of any related illnesses. My consent to the study will include this follow up.** ☐

10. **I understand that the screening process to decide if I am suitable to be selected as a participant may include completing a medical screening questionnaire and/or a physical examination by a medical officer or nurse and I consent to this.** ☐

11. **This consent is specific to the particular study described in the Participant Information Sheet and shall not be taken to imply my consent to participate in any subsequent study or deviation from that detailed here.** ☐

12. **I understand that in the event of my sustaining injury, illness or death as a direct result of participating as a volunteer in this research, I or my dependants may enter a claim with the Ministry of Defence for compensation under the provisions of the no-fault compensation scheme, details of which are attached.** ☐

Participant's Statement :

I ....

agree that the research project named above has been explained to me to my satisfaction, and I agree to take part in the study.

Signed : Date :

Investigator's Statement :

I ....

confirm that I have carefully explained the nature, demands and any foreseeable risks of the proposed research to the Participant.

Signed : Date :

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REGULATORY PROCESSES

The study has been awarded an Investigating New Drug (IND) Exemption by the FDA. It has also been given approval by the US Scientific Review Board (SRB, equivalent to UK Scientific Advisory Committees) and is awaiting review at Institutional Review Board (IRB, equivalent to UK Research and Ethics Committee.)

I am the nominated UK Protocol Director and Principal Investigator. This will require:

- Army Scientific Advisory Committee (ASAC) approval
- Ministry of Defence Research and Ethics Committee (MODREC) approval
- Medicines and Healthcare products Regulatory Agency (MHRA) waiver as it is considered a non-Clinical Trial of Investigational Medicinal Product (non-CTIMP.)

CHAPTER 5. Conclusions, discussion and future plans.

Travellers' Diarrhoea is a significant issue for overseas travellers and deploying troops. The detrimental effects have been demonstrated over many years and despite improvements in sanitation, TD incidence rates remain above 10%, which has an impact on individual routines and the operational effect.

During the PREVENT TD study, Rifaximin 550mg twice daily was proven a safe and effective antibiotic in the prevention of travellers' diarrhoea (TD) in Military personnel deployed for a 6 week period to BATUK, Kenya when compared to placebo (0.05 vs 0.36/ 100 person days, p=0.025).

Previous studies using antibiotics are prophylaxis have found high levels of AMR developing and thus antibiotics becoming ineffective. This is coupled with the increasing levels of AMR developing globally. Rifaximin is not systemically absorbed but there would still be concern about the gut microbiome developing AMR. It is reassuring to see this is not the case.

The study found high baseline levels of ESBL-E carriage in soldiers. 37/121 (30.6%) subjects commenced the study with ESBL-E in their stool samples. As this was a secondary outcome, there was insufficient information recorded in the enrolment questionnaires regarding previous travel, recent antibiotic exposure and close contact with other study participants. Comprehensive knowledge of the individuals' prior overseas travel and previous medication, especially antibiotics would have strengthened this studies as these may be contributing factors to the high level of ESBL-E carriage, when compared to previously published data. These limitations are to be addressed in a subsequent study by a military colleague who will follow a cohort of soldiers through their deployment; collecting pre-deployment information and serial stool samples during their deployment. They will also collect any diarrhoeal stool samples, which will be analysed using a real-time PCR BioFire™ film array on site at BATUK, Kenya. This will be followed by laboratory testing and genomic sequencing at United States Army Medical Research Directorate Kenya (USAMRU-K) in collaboration with Kenya Medical Research Institute (KEMRI). Amongst other MDR testing, it will also look for ESBL-E in the diarrhoeal stool samples and the findings from this study may guide future antimicrobial policy.

A caveat to comparing the study described here and future work at BATUK , is that the COVID-19 pandemic happened between the studies. It is possible that hand washing and personal hygiene standards have improved because of this, and there may be more attention to minimise crowding.

This study was underpowered to determine whether the rifaximin intervention could impact the acquisition rate of ESBL-E during deployment, but we did not find evidence for an increase in ESBL-E carriage in those taking rifaximin prophylaxis for prevention of TD in a military population deployed to BATUK, Kenya for a 6-week period.

E. coli was used as a surrogate for studying the coliform population within individuals and the effect of rifaximin prophylaxis on the rates of ESBL-E. carriage over the 6-

week study period. ESBL-E and non-ESBL-E samples for each individual were sent to MicrobesNG for genomic sequencing.

E. coli phylogroups in strains isolated are typical of gastrointestinal commensals and enteric pathogenic strains, but individual STs which are frequently associated with ExPEC were also found.

Despite the high levels of *E. coli* carrying a number of different VF, there was low levels of clinically apparent TD. 17 of the study participants suffered TD, unfortunately diarrhoeal stool samples from the time are not available, given the difficulties of collecting and storing samples commonly experienced. 8 of these individuals had paired samples, for which I was able to recover *E. coli.*

38/40 (95%) of phenotypically ESBL *E. coli* showed bla genes when using NCBI. The exceptions were PU0070_V2_REC1 and PU0143_V2_REC1 this can possibly be explained by the excretion of β-lactamase by surrounding bacteria that allow the above *E. coli* to grow, despite them not possessing their own *bla* genes.
18/38 (47.3%) of strains contained widely recognised AMR genes, with the remainder demonstrating those from the *blaEC* family. There has been further understanding of the *blaEC* gene and its role in cephalosporin resistance since the completion of the laboratory work in 2021. Since the completion of the laboratory work in 2021, development of the literature regarding the *blaEC* genes indicate their importance in cephalosporin resistance particularly (Schmidt et al. 2023).

Interestingly, 10 pairs (one grown on ESBL Chromoselect agar, the other on non-selective agar) of *E. coli* were the same ST and carried the same resistance genes on Abricate with NCBI. This suggests the ESBL *E. coli* may be the dominant strain in these individuals. Though this cannot be confidently confirmed as only a single strain was taken from each plate, this does suggest that there is a competitive evolutionary advantage in EBSL resistance under the conditions in BATUK.

To further the work on maintaining gut health and preventing TD in those travelling overseas, a further study (P2) will began recruiting in September 2022. It is a US-UK collaboration for which I am the UK PI. The study has evolved since the production of the original protocol (contained above) and the regulatory approval. It is now comparing Travelan ® (an anti-*E. coli* immunoglobulin) against placebo in the maintenance of gut health.

At the time of writing (Jan 2024), we have recruited 164 individuals out of our target of 300. The study now includes the Liverpool School of Tropical Medicine (LSTM) as a commercial partner. We will also perform a cost-effectiveness analysis and this study's finding may in time lead to policy changes for the prevention of Travellers' Diarrhoea in the military.

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APPENDICES.
APPENDIX A. Grant award from RAMC Charity.

The RAMC Char ty
Drummond and Pr ze Comm ttee
Reg menta Headquarters RAMC
FASC
S m Road
Camber ey
Surrey
GU15 4NP

Te ephone: C v   an: 01276 412790
E ma :   ramcjourna @hotma .co.uk

Major Troth RAMC
…………………..
Van Scha k Lab
Inst tute of M crob o ogy and Infect on
Co ege of Med ca and Denta Sc ences
Un vers ty of B rm ngham
Edgbaston
BIRMINGHAM
B15 2TT

Reference Troth/112019

Date: 25 November 2019

Dear Major Troth,

DRUMMOND GRANT APPLICATION

The Drummond Comm ttee met recent y and cons dered your app cat on for the RAMC Char ty to support your research project.

I am p eased to say that your app cat on was successfu and a grant of £27,750.00 was agreed. Th s does not nc ude the cost of the ap top wh ch was removed fo ow ng d scuss on w th your goodse f.

Before we can re ease the funds to you, we w   need to have the deta s of the off c a account where we can make the transfer, a ternat ve y we can make the payment by cheque. P ease note that we are unab e to make payments to nd v dua s.

A cond t on of the award s that the Comm ttee w   need to have regu ar updates on the progress and a report at the end of the project.

The Comm ttee Members w sh you every success for the future.

Yours s ncere y

Luc e Hammann
For: Cha rman, Drummond Comm ttee.

Registered Charity No 1129091

APPENDIX B. FDA IND Exemption letter, IDCRP. 23 Jul 20.

DEPARTMENT OF THE ARMY
HEADQUARTERS, U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
810 SCHREIDER STREET
FORT DETRICK, MARYLAND 21702-5000

FCMR-ORA 23 July 2020

MEMORANDUM FOR RECORD

SUBJECT: Investigational New Drug Exemption Opinion for Investigational New Drug Fiscal Year 20-007: A Randomized, Double-Blind, Placebo-Controlled Trial to Evaluate Dietary Supplements to Maintain Gut Health During Deployment and Travel

1. The protocol and its associated documentation have been reviewed against Sections 201 and 403 of the Federal Food, Drug, and Cosmetic Act and as further delineated in the U.S. Food and Drug Administration (FDA)'s December 2006 draft document titled "Guidance for Industry on Complementary and Alternative Medicine Products and Their Regulation by the Food and Drug Administration."

2. After careful review, it was determined that the clinical investigation does not meet Investigational New Drug (IND) criteria, as the products are dietary supplements that are used as such in the clinical trial. Therefore, an FDA IND application is not recommended.

3. Details of the review can be found in the attached IND Exemption Opinion Worksheet.

4. The point of contact for this action is Ms. Emily Badraslioglu at

MARK S. PAXTON, MS
FDA Sponsor's Representative
Office of the Surgeon General

APPENDIX C. MODREC approval letters.

MODREC Secretariat
Defence Science and Technology

Dst Portsdown West, Fareham, PO17 6AD
Te ephone: 0300 153 5372
E ma : DST MODRECTeam@mod.gov.uk

Dr Tom Troth
Academ c Department of M tary Med c ne
Roya Centre of Defence Med c ne (RCDM)
HQ Jo nt Med ca Group
ICT Bu d ng, B rm ngham Research Park
V ncent Dr ve
BIRMINGHAM
B15 2SQ

Te :
Ema :
et

Our Reference: 2050/MODREC/21

Date: 2nd August 2021

Dear Dr Troth,

A Randomised, Double-Blind, Placebo-Controlled Trial to Evaluate the Efficacy of Dietary Supplements to Maintain Gut Health During Deployment and Travel (P4)

Thank you for subm tt ng your rev sed app cat on (2050/MODREC/21) w th tracked changes and the cover ng etter w th deta ed responses to the MODREC etter. I can conf rm that the rev sed protoco has been g ven favourab e op n on ex Comm ttee.

Th s favourab e op n on s va d for the durat on of the research and s cond t ona upon adherence to the protoco – p ease nform the Secretar at f any amendment becomes necessary.

P ease note that under the terms of JSP 536 you are requ red to not fy the Secretar at of the commencement date of the research, and subm t annua and f na /term nat on reports to the Secretar at on comp et on of the research.

Yours s ncere y,

Dr S mon Ko stoe
MODREC Cha r