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Epidemiology, Risk Factors, Hidden Gut Resistome and Characterization of Multidrug-Resistant *Enterobacterales* Colonizing the Intestinal Tract of Swiss Expatriates

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Abstract

A study following long-term residents from countries with low prevalence of multidrug-resistant *Enterobacterales* residing in countries with high prevalence has never been done before at this scale. In this ongoing work, we analyzed the epidemiology and risk factors for colonization with multidrug-resistant *Enterobacterales* in 57 Swiss embassy employees and expatriates stationed worldwide by administering a questionnaire to establish facets of participants' diet, lifestyle, and medical history. From stool samples sent by these participants, we found 33% were colonized with extended-spectrum β-lactamase and/or AmpC-β-lactamase producing *Escherichia coli* at the intestinal level. We then characterized the molecular features of these strains using whole-genome sequencing. Various strains carried *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{DHA-1}}$, *qnr*, and other resistance genes. Four strains also belonged to the pandemic clone of sequence type 131. We characterized the hidden gut resistome using a shotgun metagenomics approach and found that the hidden gut resistome is a reservoir for many antibiotic resistance genes. Surprisingly, we found no clear difference in the hidden gut resistome between participants colonized by multidrug-resistant *Enterobacterales* and non-colonized participants, and we were unable to reliably identify multidrug-resistant *Enterobacterales* using this method. Lastly, we identified the most significant risk factors for colonization with multidrug-resistant *Enterobacterales* were: use of antibiotics in the last 2 years, diarrhea in the last 3 months, individuals aged 36-50, being stationed in an embassy in Africa or Asia, and international travel to Africa or Asia. This study provides preliminary results for a previously unexplored population and will aid in ameliorating the problem of antibiotic resistance worldwide.

Key words:

Multidrug-resistant, Antibiotic, *Enterobacterales*, ESBL, Plasmids, Risk Factor

Popular Scientific Summary:

In 1928 when the famous Alexander Fleming first discovered the antibacterial effects of penicillin, the world would never again be the same. Antibiotics revolutionized the way people thought about medicine and how they interacted with the world around them. Infected cuts, needing emergency and basic surgery, and other bacterial infections were no longer death sentences. The production of the first antibiotic drugs even revolutionized the way human beings engaged in warfare. In short, the effects that antibiotic drugs have had on global health and modern societies are innumerable. However, the healthcare utopia driven by antibiotics is at huge risk of soon crumbling. While human beings have enjoyed the revolution that antibiotic drugs permitted them, bacteria have been evolving and communicating to return to their former glory.

One largely important environment for these interactions and communications is in the human gut, so called the gut microbiome. Knowledge in the field of the gut microbiome has increased rapidly in the last decade. We are learning more about the benefits this environment can have on human health, but we have also learned about the potential dangers of this environment. It turns out that bacteria in the gut have always been communicating with each other. These bacteria are constantly sharing genetic information on how to survive better in changing environments such as those with antibiotics. Unfortunately for human beings, these communications mean bacteria are getting better and better at resisting our antibiotic drugs.

Scientists now believe that the gut microbiome may be a perfect environment for a lot of these communications to occur between many bacterial species (**Figure 1**). This is concerning because the field of global health may once again have to fear dangerous bacteria just as it did one century ago. Nevertheless, scientists around the world are working tirelessly to be one step ahead of the bacteria and their growing genetic arsenal. By looking at a group of Swiss expatriates based throughout the world our project provides more information on the risk factors of getting infected by these resistant gut bacteria and we determined what genetic factors make these bacteria particularly efficient at resisting our antibiotic drugs. Moreover, we characterized the genetic information of bacteria that scientists have previously been unable to grow in the lab and found many antibiotic resistant genes in this environment. Lastly, we determined how widespread resistant bacteria and antibiotic resistant genes are throughout the world. In learning more about the gut microbiome, scientists hope to give the world a fighting chance in the everchanging landscape that is the battle between humans and bacteria.

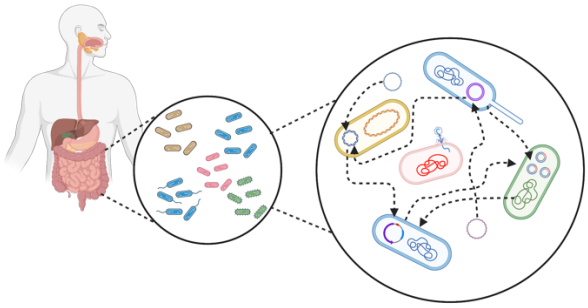

Figure 1. Representation of the gut microbiome environment where bacterial species are sharing plasmids, genes, insertion sequences and transposons. Created using Biorender.com.

Introduction:

Antibiotic resistance is a major global health problem and one of the major concerns in the One Health perspective [1, 2]. *Enterobacterales* such as *Escherichia coli* and *Klebsiella pneumoniae* contribute largely to the impact of this global health problem [1]. Many of the species which contain various antibiotic resistance mechanisms are multidrug-resistant (MDR), and therefore able to reduce the effectiveness of antibiotic drugs from many known classes [1]. Moreover, such increases in resistance can significantly lower the efficacy of life-saving antibiotics (e.g., third-generation cephalosporins (3GC), carbapenems, and polymyxins) which further exacerbates this global health problem [1, 3-5]. Additionally, certain species of *Enterobacteriaceae* such as *Proteus spp., Serratia spp., Providencia spp.,* and *Morganella morganii* are intrinsically resistant to polymyxins [6]. These species are also often associated with nosocomial outbreaks of resistant bacteria making the task of treating MDR bacteria even more complex [6, 7].

Recently, increasing evidence suggests that MDR bacteria are becoming a growing problem in the gut microbiome environment [1]. The gut microbiome is an environment where diverse groups of *Enterobacterales*, among many other groups of bacteria, are in constant contact and competition with each other [8]. While many *Enterobacterales*, including *E. coli*, exist commensally within the human host, they often carry antibiotic resistance genes (ARGs) [8-10]. These ARGs can be located within bacterial chromosomes or within transmissible mobile genetic elements (MGEs) such as plasmids [8-10]. During antibiotic treatment, ARGs can be highly expressed and may lead to difficult-to-treat infections, or to propagating these ARGs into hospital and community contacts [8-10]. Significant ARGs such as $bla_{\text{CTX-M-15}}$ and $bla_{\text{DHA-1}}$ which express extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases, respectively, are largely responsible for resistance of *Enterobacterales* species to many clinically relevant β-lactam antibiotics [7, 11]. Global dissemination of these genes has further complicated the worldwide health challenge of treating and halting the spread of MDR bacteria [2, 7, 11-14]. Furthermore, antibiotic use increases the evolutionary pressure for chromosomal mutations and facilitates the exchange of resistance mechanisms through MGEs (**Figure 1**) [11, 15]. Moreover, *Enterobacterales* such as *E. coli* carry many other resistance mechanisms such as the multidrug AcrAB efflux pump and selectively permeable membrane porins which lower the efficacy of drugs acting within bacterial cells [7]. Therefore, it is essential to further characterize the organisms which are capable of causing infections within the human gut, and in extraintestinal tissues [7]. This may be especially problematic in cases of sepsis, hospital-acquired pneumonia, urinary tract and surgical site infections which have been previously linked to MDR *Enterobacterales* (MDRE) [7].

It has previously been established that nosocomial spread of MDRE contributes greatly to the global epidemiology of these resistant isolates, revealing that hospitalizations are a significant risk factor for colonization with MDRE [5, 7]. However, this is not the only risk factor for colonization with these organisms. One study explored the anthropological and socioeconomic factors that are involved in the spread of MDR organisms (MDROs) and revealed that global antibiotic resistance is related to quality of governance, access to clean water, and public health spending [13]. Moreover, recent studies have revealed that irritable bowel disease, antibiotic use within the last 3-12 months, and international travel destination are all significant risk factors for colonization with MDRE [5, 8-10, 12, 16, 17]. This reveals that battling antibiotic resistance worldwide extends beyond the lab and requires extensive interdisciplinary collaboration in the One Health perspective to improve the current global situation [2, 13].

Notably, travelers from high-income to low and middle-income countries may be at particular risk of being colonized by the MDRE that are present in local communities and

hospitals [5, 8-10, 12, 16, 17]. Moreover, these travelers often carry these MDROs and their ARGs back to their home countries when they return from their travels and are at risk of propagating these organisms within their communities or developing difficult-to-treat infections themselves [8-10, 12, 17]. A recent study following travelers from Switzerland to the island of Zanzibar (Tanzania) showed that around 54.1% of study participants (20/37) who were not previously colonized with extended-spectrum cephalosporin-resistant (ESC-R) bacteria likely acquired new colonization on their particular trip to Zanzibar [18]. Similarly, a study looking at 170 travelers from Switzerland to the region of South Asia demonstrated that 69.4% of all travelers were colonization with ESBL-producing *Enterobacterales* during their time in South Asia [10]. In the same study, colonization also varied by travel destination where 86.8% of travelers to India developed colonization with MDRE, while only 34.7% of travelers to Sri Lanka developed colonization with MDRE indicating that travel destination is a relevant risk factor for colonization [10]. Although there is some variation between travel destination it is clear that international travel to low and middle-income countries is a significant risk factor for colonization with MDRE [8, 10, 12, 13, 17]. Furthermore, there is increasing evidence that international travel can be attributed to the importation of the *mcr-1* gene to Switzerland which is responsible for colistin resistance and has been found in plasmids carried by international travelers [17]. Moreover, acquiring new colonization during travel and extended stays in foreign countries increases the risk for further propagation of MDROs to community and hospital contacts, particularly in cases of previous or ongoing antibiotic treatment [1, 5, 9, 10, 16-18]. There is still much work to be done to fully characterize the organisms and the level of spread of MDRE during extended stays in low, middle, and high-income countries and in countries with varying prevalence of MDRE colonization. This study aims to address some of these concerns.

Generally, bacterial strains isolated from stool samples are grown in non-selective media and then used for selective plating and minimum inhibitory concentration (MIC) determination to assess their extent of antibiotic resistance. Occasionally, MDROs are missed under this protocol due to the lack of selection for antibiotic resistant organisms [16]. In this study, we utilized a broth enrichment method to culture enteric microorganisms in antibiotic rich media to increase the likelihood of detection of MDRE. This protocol should more accurately determine the MDRE colonization status of our study participants which will be analyzed to establish the epidemiological risk factors for colonization in this underreported population.

In this work, we speculate that Swiss Embassy Employees (EEs), their household contacts such as relatives and partners (RP), and other Swiss expatriates stationed throughout the world may be at comparable risk to international travelers for being infected with endemic MDRE. We also speculate that they may be at risk for spreading MDROs in Switzerland upon their return. Furthermore, due to their extended stays in MDRE-endemic countries, we believe that Swiss expats may be colonized at comparable rates, and by similar MDRE as the local population. These data will provide epidemiological information on a previously underreported population and will contribute to monitoring the spread of MDRE throughout the world. Indeed, a study looking at MDRE colonization in long-term international residents such as Swiss Embassy employees and expats has never been done at the scale projected for this study. Studying these specific populations may reveal valuable information on the global dissemination and infection dynamics of MDRE. Moreover, by studying the hidden gut resistome in these Swiss expats we will more accurately characterize the gut microbiome environment as a potential reservoir for many ARGs of particular clinical and epidemiological relevance. In turn, intervention strategies can be more effectively implemented to address this major global health problem.

Aims of the project:

In this study, we aimed to characterize the antibiotic resistance phenotypes and ARGs that make MDRE particularly dangerous from the One Health perspective [2]. This was done through MIC determination, and both short and long read DNA sequencing. Furthermore, we aimed to apply a shotgun metagenomics protocol to detect ARGs in the gut microbiome to characterize the hidden gut resistome from unculturable microorganisms. Most significantly, we aimed to characterize and outline the risk factors for gut colonization with MDREs to help ameliorate this global health issue.

Materials and Methods:

1.1 Ethical considerations

Ethical considerations for this study were supported by Prof. Parham Sendi. Ethical clearance was obtained by the *Kantonale Ethikkommission für die Forschung*, KEK, Bern (BASEC-Nr. 2020-01683).

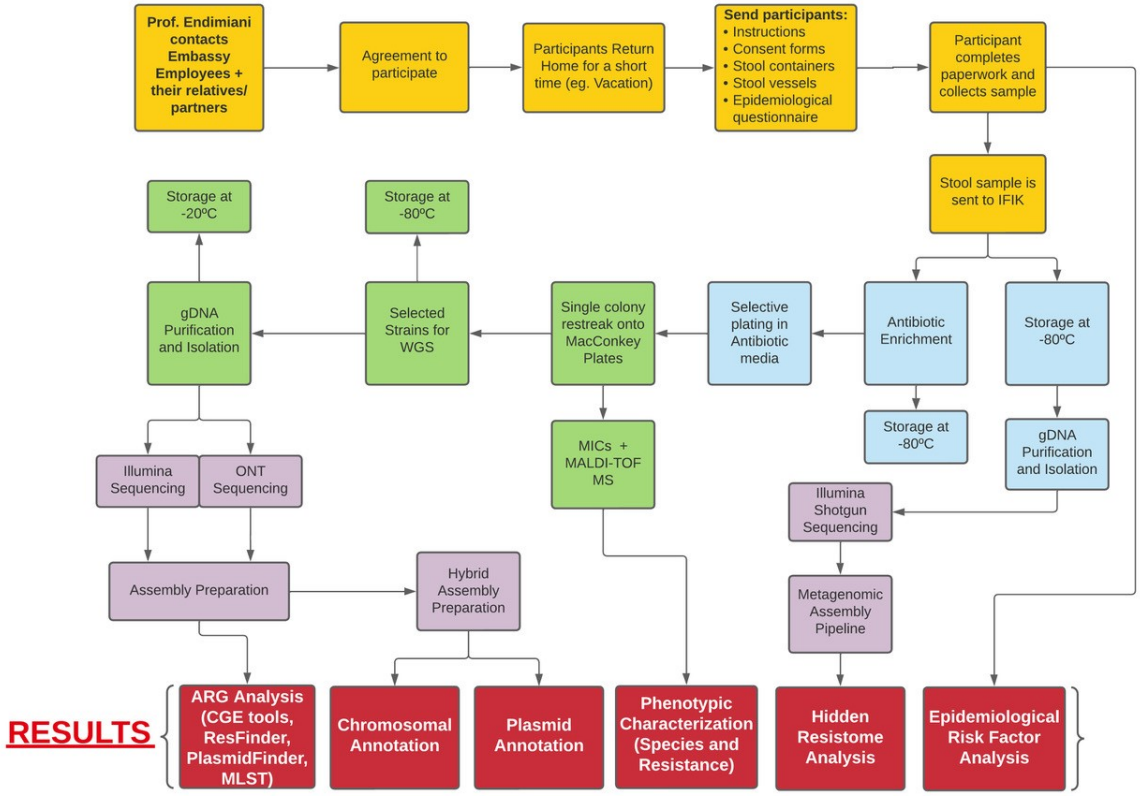

Figure 2: Study outline and intended results. Institute for Infectious Diseases, IFIK; genomic DNA, gDNA; minimum inhibitory concentrations, MICs; Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectroscopy, MALDI-TOF MS; Whole Genome Sequencing, WGS; Oxford Nanopore Technology, ONT; Center for Genomic Epidemiology, CGE; Antibiotic Resistance Genes, ARGs; Multi-locus Sequence Typing, MLST. Created with Lucidchart.com.

1.2 Study overview and epidemiological questionnaire

This study was performed with the support of the Swiss Federal Department of Foreign Affairs (FDFA; https://www.eda.admin.ch/eda/en/home.html). Swiss embassies around the world (n ~102) were contacted by Prof. Endimiani and offered the opportunity to participate in the "Superbugs Study" to identify potential MDRE in their intestinal tracts (see also https://www.ifik.unibe.ch/research/gram_negatives___andrea_endimiani/join_superbugs_stu

dy/index_eng.html). Volunteers were then provided with a questionnaire designed to identify potential risk factors and predisposing conditions for either carrying or acquiring MDRE. Epidemiological data, data validation, quality control, participant anonymization, and public data sharing were performed by the Clinical Trials Unit (CTU) Bern (https://www.ctu.unibe.ch/) and stored in the Research Electronic Data Capture (REDCap) database (https://www.project-redcap.org/). The epidemiological questionnaire was used to quantify the significance of predisposing risk that volunteers had in their lives abroad by asking questions pertaining to their employment status, location of embassy, diet, lifestyle, previous hospitalizations, antibiotic use, and proximity to pets and other animals (see **Table 6**). Volunteers were then sent sample collection vessels, written consent forms, and instructions on submitting stool samples to the Endimiani Group at the Institute for Infectious Diseases (IFIK) in Bern, Switzerland. Within one week of volunteers' return to Switzerland, stool samples were collected in specific vessels and sent to IFIK using the ordinary postal service. See **Figure 2** for a general outline of the study and its intended results. This study is still ongoing and so far, 57 healthy Swiss expatriates from 31 Swiss embassies have been included. Optimally, by the end of the study, ~250 participants from ~102 Swiss embassies will be included in the study.

1.3 Statistical Analysis

Responses to the epidemiological questionnaire were archived and evaluated based on participant colonization status with MDRE. Each predisposing condition or risk factor was evaluated separately in a 2x2 table and odds ratios (OR) were calculated from the inputs as shown below (see also **Table 6**). Note: OR for MDRE colonization based on geographical location were compared to Europe as a baseline due to literature evidence that Europe is the region with the lowest relative prevalence of MDRE colonization worldwide [19, 20]. Confidence intervals (CI) were calculated in Excel with the below formula as previously described where "*e*" is the mathematical constant ≈ 2.718, OR is the calculated odds ratio, 1.96 is the critical value of the normal distribution for calculating a 95% confidence interval; "*a-d*" are the inputs to the 2x2 table [21]:

Table 1: 2x2 table format used for OR and CI calculations.

	Colonized with MDRE	Not Colonized with MDRE	TOTALS
Group 1	*a*	*b*	*a+b*
Group 2	*c*	*d*	*c+d*
TOTALS	*a+c*	*b+d*	*a+b+c+d*

$$Odds\ Ratio = ad/bc$$

$$Confidence\ interval = e^{(\ln(OR) \pm 1.96\sqrt{(1/a + 1/b + 1/c + 1/d)})}$$

2.0 Stool Screening

Each stool sample was received and immediately processed into two liquid antibiotic enrichments. A colistin enrichment tube was prepared by adding one 10 µg colistin CL-10 disc into a tube containing 10 mL of Luria Bertani (LB) broth [1 mg/L]. Similarly, a cefuroxime enrichment was prepared by adding one 30 µg cefuroxime CMX-30 disc into 10 mL of LB broth [3 mg/L]. Around 50 µg of participant's stool was then added to each of the enrichment tubes. Tubes were incubated overnight at 36ºC (±1°) in the shaking incubator.

Overnight cultures were diluted in a 0.85% NaCl solution to obtain a 0.5 McFarland solution (~1.5 x 10^8 CFU/mL) on the Densicheck-reader (bioMérieux). From the colistin enrichment 100 µL of the 0.5 McFarland solution was plated onto a CHROMID COL-R plate (bioMérieux) and from the cefuroxime enrichment 100µL of the 0.5 McFarland solution was plated onto CHROMID ESBL and CHROMID CARBA Smart plates (bioMérieux). All plates were then incubated overnight at 36ºC (±1°).
Morphologically different colonies were regrown overnight at 36ºC (±1°) on MacConkey plates and used for matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) (Bruker) species identification (ID), antimicrobial susceptibility tests (ASTs), genomic DNA extraction, and to prepare glycerol stocks for optional future analysis. Species naturally resistant to polymyxins growing on CHROMID COL-R plates were excluded from further analyses as described above.
Storage: stool aliquots samples were prepared and stored at -80ºC in freezer-safe tubes (Cryo.s, Greiner Bio-One). Enrichment aliquots were stored in 20% glycerol at -80ºC in the same freezer-safe tubes. Isolated strains were stored at -80ºC by preparing stocks using the TS/80-YE Project Microorganism Preservation System (Technical Service Consultants Ltd).

3.0 Species identification (ID)

Individual colonies were selected from the overnight MacConkey plates from the last step of the Stool Screening protocol for MALDI-TOF MS ID. Individual colonies were taken up with a toothpick and smeared onto a free spot on the MALDI-TOF MS target plate. One µL of formic acid was added to each spot and allowed to dry completely. After drying, 1 µL of alpha-cyano-4-hydroxy-cinnamic acid (CHCA; Bruker) matrix was added to each spot, and again allowed to dry completely. The MALDI-TOF MS plate was added to the MALDI-TOF MS machine (Bruker Microflex LT) and run according to the MALDI Biotyper® System (Bruker). Output results were documented and archived. Results from the MALDI-TOF MS ID were then confirmed using the Type Strain Genome Server (https://tygs.dsmz.de/) and the JSpeciesWS online service for species identification (http://jspecies.ribohost.com/jspeciesws/) using whole genome sequencing draft and complete assemblies (see below, sections 4.1-4.3).

3.1 Antimicrobial susceptibility tests (ASTs)

For the ASTs, 2 mL of NaCl were added to a Falcon-tube (Corning Science Mexico). Using a cotton swab, colonies were taken from overnight cultures of the last step in the Stool Screening protocol. Colonies were mixed well into the 2 mL NaCl solution until a 0.5 McFarland was confirmed on the Densicheck-reader. Ten µL of the 0.5 McFarland solution was added to a 10 mL cation-adjusted Müller Hinton broth (CAMHB) culture tube. Fifty µL of the CAMBH-bacteria solution was added to each well of a Sensititre™ GNX2F 96-well plate (Thermo Fischer Scientific) and incubated overnight at 36ºC (±1°).
AST results were interpreted as susceptible (S), susceptible; increased exposure (I), or resistant (R) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2022 guidelines (v12.0) (https://www.eucast.org/clinical_breakpoints/). *Enterobacterales* strains which were classified as 'I' or 'R' to penicillins and 3GC, and/or carbapenems, and/or colistin were included in the study for further analysis.

4.0 Genomic DNA

Genomic DNA from suspected MDRE colonies was extracted and purified according to the PureLink™ Microbiome DNA Purification Kit protocol for microbial DNA from culture media (Invitrogen by ThermoFisher Scientific). Purified genomic DNA samples were evaluated and quantified using a Qubit 3.0 reader and Nanodrop Spectrophotometer (Thermo Fisher

Scientific). (See section 5.0 for information regarding metagenomic DNA isolation from stools).

4.1 Illumina Whole-Genome Sequencing (WGS)

DNA paired-end reads (2 x 150 bp read lengths) for WGS were produced using the NovaSeq 6000 sequencing platform performed by Eurofins genomics (https://eurofinsgenomics.eu/) as previously described [22]. Illumina reads were filtered with the Trimmomatic (v0.36) trimmer to generate fastq sequence reads. This was followed by whole genome assembly using Unicycler (v0.4.8). Assembled reads were polished for several cycles using the Pilon programme (v1.22). Draft assemblies from Illumina SPAdes assemblies were used for whole-genome identification using the Center for Genomic Epidemiology online tools (CGE; http://www.genomicepidemiology.org/): ResFinder (v4.1), MLST (v2.0), and PlasmidFinder (v2.0) as previously described [12, 22].

4.2 Oxford Nanopore Technology (ONT)s: MinION Sequencing

Sequencing libraries were prepared in-house using the "Rapid Barcoding Sequencing (SQK-RBK004) protocol" from Oxford Nanopore Technologies (version: RBK_9054_v2_revR_14Aug2019) and added to a MinION sequencer (Oxford Nanopore Technologies). The libraries were allowed to run for 48 hours with basecalling switched off. Basecalling was performed using the Guppy GPU (v3.4.5) using the High Accuracy model (HA) outlined by Oxford Nanopore Technologies.

4.3 Hybrid Assemblies and Plasmid Annotations

Hybrid assemblies were generated with Unicycler (v0.4.8; hybrid pipeline; default parameters); independent long-read only assemblies were generated with Flye (v2.9, 5 polish iterations) to compare and assess the quality of the Unicycler assemblies. Long-read quality filtering was performed using Porechop (v0.2.4) with default settings; and Filtlong (v0.2.0) using Phred quality scores; with 1 billion target bases. Hybrid assemblies were then evaluated using the CGE tools as previously described, and then separated into contiguous fragments to be distinguished between plasmid or chromosomal reads [12, 22].
Plasmids containing ARGs based on CGE results were subjected to NCBI's BLAST feature and hits were evaluated for sequence similarity. Sequences with acceptable query cover and sequence identity (>90%) were selected as references for plasmid annotations. If no acceptable reference sequences were found, annotations were prepared using Prokka (v1.13) with default settings from plasmid contiguous fragments. Insertion sequence (IS) elements were annotated manually with ISfinder (https://isfinder.biotoul.fr/). Plasmids were then grouped based on incompatibility groups and aligned using the Blast Ring Image Generator (BRIG) software (v0.95) with previously selected or generated reference sequences. NCBI or Prokka annotations were then used to annotate the plasmid figures for ARGs, MGEs, and other resistance factors.

5.0 Metagenomic Sequencing

Genomic DNA was extracted and purified directly from stool samples of interest according to the PureLink™ Microbiome DNA Purification Kit protocol for microbial DNA from stool samples (Invitrogen by Thermo Fisher Scientific). Purified genomic DNA was sent to Eurofins Genomics (https://eurofinsgenomics.eu/) for metagenomic sequencing. In brief, the INVIEW Metagenome protocol for the NovaSeq 6000 S2 PE150 XP Illumina genomics system was used. Standard genomic 150 bp paired-end library preparation (Nextera XT DNA Library Prep

kit) with unique dual indexing was used by Eurofins. Fastq files coupled with sequences and quality scores were delivered to the Endimiani lab for further in-house analysis. In the Endimiani lab, quality checks were performed using FastQC (v0.11.9). Adaptor sequences (minimum length set to 50 bp) were removed from the paired-end reads and quality-filtered with Trimmomatic (v0.36). Once more, a quality check was performed using FastQC (v0.11.9). Host reads (*Homo sapiens*) were first removed with Kneaddata (v0.10.0) using the GRCh37 and GRCh38 (with decoy) human genome assemblies; followed by Kraken2 (v2.1.1) decontamination using the GRCh38.p13 human genome. Metagenomic assemblies were prepared from Illumina reads with MetaSpades (v3.14.0) under default settings. Final assembly ARG screening was conducted with Abricate (v1.0.1) using the ResFinder database.

Results:

33% of Study Participants were Colonized with MDR *E. coli*

Enriched stool samples were plated on selective media and identified by MALDI-TOF MS as described above to identify the presence of MDRE. We found that 33% (19/57) of the study participants were colonized with MDR *E. coli* (see **Table 2** for detailed output of study participants). No further *Enterobacterales* with an MDR phenotype were included in this study.

African Region:

37% (7/19) of the identified MDRE-colonized participants were stationed in the African region (**Figure 3**). Four of the MDRE-colonized participants based in Africa (S1-KEN-03, S1-KEN-04, S1-KEN-07, S1-ZIM-01) were classified as EEs, and one (S1-IVC-02) was classified as a RP. One of the colonized individuals was classified as a Swiss expat (S1-KEN-05) who also had a colonized relative (S1-KEN-06) with a different MDR *E. coli* (**Table 4**). This may change in future screenings.

European Region:

In the European region we found 16% (3/19) of the MDRE-colonized study participants (S1-ESP-02, S1-ESP-03, S1-SRB-02) (**Figure 3**). All of these study participants were classified as EEs; S1-ESP-02 and S1-ESP-03 were associated (they worked in the same embassy), but were colonized with different MDR *E. coli*. Interestingly, S1-SRB-02 shared no relation to S1-ESP-02 and S1-ESP-03, but was colonized by an MDR *E. coli* with the same sequence type (ST), chromosomal ARGs and plasmids as S1-ESP-03 (**Table 4**).

Asian Region:

In the Asian region we found 37% (7/19) of the MDRE-colonized study participants (**Figure 3**). Six of the MDRE-colonized participants (S1-ISR-02, S1-CHI-01, S1-CAM-02, S1-IND-01, S1-IND-02, S1-SRL-01) were classified as EEs while one MDRE-colonized participant (S1-CAM-03) was the relative of the EE S1-CAM-02. All other participants from the Asian region had no relation to each other.

American Region:

Lastly, in the American region (mostly South America) we found 10% (2/19) study participants (S1-VEN-01, S1-HAI-01) were colonized with MDRE (**Figure 3**). Both of these participants were classified as EEs and had no relation to each other.

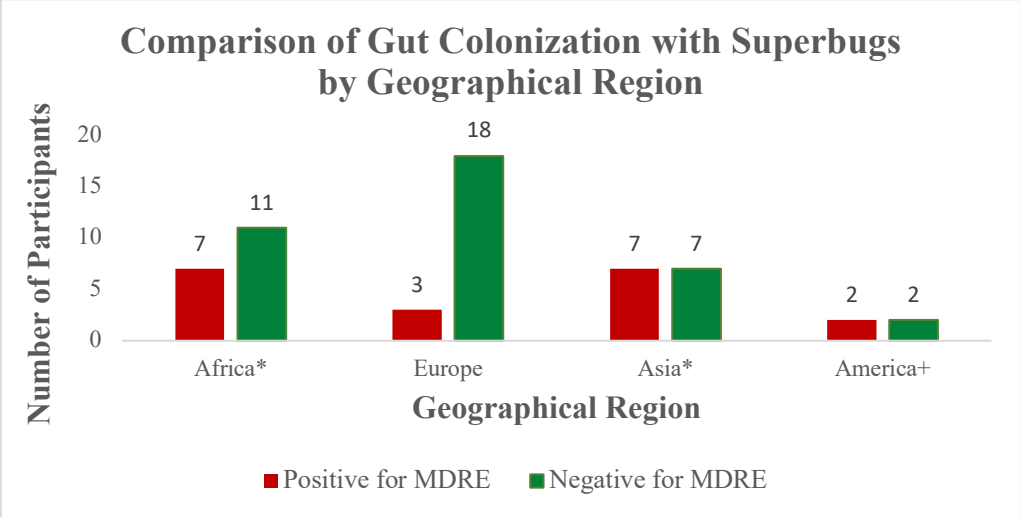

Figure 3: Number of study participants colonized by MDRE vs. Geographical Region. "*" Indicates increased risk of colonization as compared to the European region included in the study (n=57). "+" Indicates that a comparison could not be made due to a limited number of samples.

Table 2: General characteristics of study participants including their MDRE colonization status (n=57).

Participant ID	Study Classification	Age	Sex	Household Contact	Geographical Region	MDRE Colonization Status
S1-KEN-01	EE	26	M	-	**Africa** (Kenya)	-
S1-KEN-03	EE	40	F	-	**Africa** (Kenya)	+ MDR *E. coli*
S1-KEN-04	EE	59	M	-	**Africa** (Kenya)	+ MDR *E. coli*
S1-KEN-05	Expat	39	M	**S1-KEN-06**	**Africa** (Kenya)	+ MDR *E. coli*
S1-KEN-06	Relative	31	F	**S1-KEN-05**	**Africa** (Kenya)	+ MDR *E. coli*
S1-KEN-07	EE	58	M	**S1-KEN-08**	**Africa** (Kenya)	+ MDR *E. coli*
S1-KEN-08	Relative	44	M	**S1-KEN-07**	**Africa** (Kenya)	-
S1-TAN-01	Relative	55	F	**S1-TAN-02**	**Africa** (Tanzania)	-
S1-TAN-02	EE	54	M	**S1-TAN-01**	**Africa** (Tanzania)	-
S1-GHA-01	EE	32	F	-	**Africa** (Ghana)	-
S1-BUR-01	EE	30	F	-	**Africa** (Burkina Faso)	-
S1-MOZ-01	EE	46	M	-	**Africa** (Mozambique)	-
S1-ZIM-01	EE	64	M	-	**Africa** (Zimbabwe)	+ MDR *E. coli*
S1-RSA-01	EE	60	M	-	**Africa** (Rep. S. Africa)	-
S1-RSA-02	EE	59	M	-	**Africa** (Rep. S. Africa)	-
S1-IVC-01	EE	50	M	**S1-IVC-02**	**Africa** (Ivory Coast)	-
S1-IVC-02	RP	50	F	**S1-IVC-01**	**Africa** (Ivory Coast)	+ MDR *E. coli*
S1-EGY-01	EE	54	M	-	**Africa** (Egypt)	-
S1-ESP-01	EE	39	F	-	**Europe** (Spain)	-
S1-ESP-02	EE	25	F	-	**Europe** (Spain)	+ MDR *E. coli*
S1-ESP-03	EE	51	F	-	**Europe** (Spain)	+ MDR *E. coli*
S1-GER-01	Relative	54	M	**S1-GER-02**	**Europe** (Germany)	-
S1-GER-02	EE	56	M	**S1-GER-01**	**Europe** (Germany)	-
S1-GER-03	EE	34	M	-	**Europe** (Germany)	-
S1-GER-04	EE	32	F	-	**Europe** (Germany)	-
S1-GER-05	EE	63	M	**S1-GER-06**	**Europe** (Germany)	-
S1-GER-06	Relative	60	F	**S1-GER-05**	**Europe** (Germany)	-
S1-GER-08	EE	27	F	-	**Europe** (Germany)	-
S1-ITA-01	EE	31	F	-	**Europe** (Italy)	-
S1-SRB-01	EE	62	M	**S1-SRB-02**	**Europe** (Serbia)	-
S1-SRB-02	Relative	62	F	**S1-SRB-01**	**Europe** (Serbia)	+ MDR *E. coli*
S1-KOS-02	EE	38	F	-	**Europe** (Kosovo)	-
S1-KOS-03	EE	60	M	-	**Europe** (Kosovo)	-
S1-GBR-01	EE	55	F	-	**Europe** (Great Britain)	-
S1-GBR-03	EE	57	M	-	**Europe** (Great Britain)	-
S1-BEL-01	EE	53	F	-	**Europe** (Belgium)	-
S1-POR-01	EE	45	F	-	**Europe** (Portugal)	-
S1-UKR-01	EE	35	F	-	**Europe** (Ukraine)	-
S1-NED-01	EE	31	F	-	**Europe** (Netherlands)	-
S1-CAN-01	EE	52	M	-	**America** (Canada)	-
S1-VEN-01	EE	47	M	**S1-VEN-02**	**America** (Venezuela)	+ MDR *E. coli*
S1-VEN-02	Relative	43	M	**S1-VEN-01**	**America** (Venezuela)	-
S1-HAI-01	EE	36	M	-	**America** (Haiti)	+ MDR *E. coli*
S1-NNN-01	EE	56	M	-	**Asia** (U.A.E.)	-
S1-ISR-01	EE	55	F	-	**Asia** (Israel)	-
S1-ISR-02	EE	52	M	-	**Asia** (Israel)	+ MDR *E. coli*
S1-CHI-01	EE	56	F	-	**Asia** (China)	+ MDR *E. coli*
S1-HKG-01	EE	58	M	-	**Asia** (Hong Kong)	-
S1-THA-01	Relative	57	M	**S1-THA-02**	**Asia** (Thailand)	+ MDR *E. coli*
S1-THA-02	EE	56	F	**S1-THA-01**	**Asia** (Thailand)	-
S1-THA-03	EE	60	F	-	**Asia** (Thailand)	-
S1-INA-01	EE	59	F	-	**Asia** (Indonesia)	-
S1-CAM-02	EE	48	M	**S1-CAM-03**	**Asia** (Cambodia)	+ MDR *E. coli*
S1-CAM-03	Relative	50	F	**S1-CAM-02**	**Asia** (Cambodia)	-
S1-IND-01	EE	42	M	-	**Asia** (India)	+ MDR *E. coli*
S1-IND-02	EE	45	F	-	**Asia** (India)	+ MDR *E. coli*
S1-SRL-01	EE	63	M	-	**Asia** (Sri Lanka)	+ MDR *E. coli*

Embassy Employees (EE; Employed at a Swiss Embassy), Relative (share a foreign household with an EE), Expat (Swiss citizens currently living abroad). "Household Contact" column lists the other study participant(s), if any, that share a household with each other.

Phenotypic Characteristics of Identified MDR *E. coli*

24 MDRE from the 19 colonized study participants (average of 1.26 MDRE per person) were selected for MIC analyses and were evaluated according to the EUCAST guidelines for *Enterobacterales* as described above (See **Table 3** for a detailed MIC report). The MIC analyses showed that all (24/24) study isolates tested were not susceptible to the 3GC cefotaxime. Similarly, 19/24 of the study isolates were not susceptible to the 3GC ceftazidime. Alternatively, only 7/24 study isolates were not susceptible to the 4th generation cephalosporin (4GC) cefepime. Of the 24 study isolates that were not susceptible to cefotaxime (3GC), 20 were also not susceptible to the monobactam aztreonam. All study isolates were susceptible to the carbapenem antibiotics (e.g., meropenem, imipenem). These results show that all of the study isolates demonstrate an ESBL phenotype regardless of country of residence [23, 24].

Interestingly, isolate S1-SRL-01-C was resistant to the 3GCs, aztreonam, and the penicillin/β-lactamase inhibitor combinations piperacillin/tazobactam and ticarcillin/clavulanic acid, but susceptible to 4GCs (cefepime) and the carbapenems. This indicates that isolate S1-SRL-01-C produces an AmpC enzyme with powerful β-lactamase activity (**Table 4**) [25]. Lastly, 4/24 of the study isolates were not susceptible to gentamicin and 14/24 were not susceptible to ciprofloxacin.

WGS Analysis of the MDRE

Whole genome characterization of MDRE was performed on 20 isolates selected through the stool screening protocol. Characterization was done using hybrid assemblies from ONT and Illumina assemblies as described above (**Table 4**: full list of identified ARGs). We found that 18/20 of the sequenced isolates contained an ESBL-producing $bla_{\text{CTX-M}}$ gene. Of these 18 isolates, 14 were identified as carrying the widespread $bla_{\text{CTX-M-15}}$ gene. In 7 isolates $bla_{\text{CTX-M-15}}$ was located in plasmids while the other 7 carried $bla_{\text{CTX-M-15}}$ chromosomally. Alarmingly, we identified 3/20 isolates contained $bla_{\text{DHA-1}}$. In two strains, $bla_{\text{DHA-1}}$ was located in the chromosome while one strain contained $bla_{\text{DHA-1}}$ in a plasmid. Significantly, $bla_{\text{DHA-1}}$ has previously been characterized as a pandemic plasmid-mediated AmpC-producing gene [25]. Lastly, 5/20 isolates contained $bla_{\text{TEM-like}}$ genes and 1/20 isolates contained a $bla_{\text{OXA-1}}$ gene which is characterized in resistance to the β-lactamase inhibitor combinations [26].

We identified various strains (S1-IND-01-B, S1-VEN-01-A, S1-KEN-05-A, and S1-THA-01-A) belonging to the globally disseminated ST 131 clone (**Table 4**). Each of the strains belonging to ST 131 contained a $bla_{\text{CTX-M}}$ gene which has been previously characterized in the global spread of these resistant isolates [27].

Furthermore, we identified that 14/20 of the analyzed isolates co-harbored genes that confer resistance to aminoglycoside antibiotics (*aac(3)-IIa*, and/or *aac(6')-Ib-cr*, and/or *aph(3'')-Ib*, and/or *aph(6)-Id*) [28]. Lastly, we identified 10/20 isolates harbored genes that confer resistance to fluoroquinolone antibiotics (*qnrS1* and/or *qnrB4*), and 16/20 isolates contained genes for tetracycline resistance (*tet*(A) or *tet*(B)) [29, 30].

Among the ARG-containing plasmids, we identified the most recurrent replicons (in 14/20 isolates) were FII, FIA, and FIB, which are all associated with the IncF group [31]. S1-CHI-01-A, S1-ESP-03-A, and S1-SRB-02-B carried an IncFII plasmid with $bla_{\text{CTX-M-15}}$ and *qnrS1* (**Figure 4.1**). Less commonly, we found 9/20 of the strains carried Col replicon plasmids, and were mostly found together with other plasmid replicon groups (mainly IncF). In 4/20 strains we identified IncB/O/K/Z plasmid replicons. S1-KEN-03-A, S1-KEN-03-C, and S1-KEN-04-A carried an IncB/O/K/Z plasmid with $bla_{\text{CTX-M-15}}$ and genes for aminoglycoside resistance (**Figure 4.2**). Furthermore, we identified the presence of two IncQ plasmid replicons together with other IncF plasmids. Finally, we identified one IncI plasmid in the S1-ISR-02-A strain which carried $bla_{\text{CTX-M-55}}$ (**Table 4**).

Table 3: Minimum inhibitory concentrations (MIC) in mg/L of *E. coli* strains isolated from stools of study participants (n=24). All MICs were interpreted using EUCAST guidelines.

		MIC (in mg/L) of Antibiotics from Diverse Classes																		
Strain ID	MALDI-TOF MS ID	β-lactamase Inhibitor Combinations		3/4 Cephalosporins			Mono-bactams	Carbapenems				Aminoglycosides			Fluoroquinolones		Glycyl cycline	Anti-folate	Poly-myxin	Tetra-cycline
		P/T4	TIM2	FOT	TAZ	FEP	AZT	IMI	MERO	DOR	ETP	GEN	TOB	AMI	CIP	LEVO	TGC	SXT	COL	DOX
S1-KEN-03-A	*E. coli*	≤8/4	≤16/2	8	≤1	≤1	4	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	>4/76	≤0.25	8
S1-KEN-03-C	*E. coli*	≤8/4	≤16/2	32	8	≤1	16	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	>4/76	≤0.25	8
S1-KEN-04-A	*E. coli*	≤8/4	≤16/2	8	2	≤1	4	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	0.5	>4/76	≤0.25	16
S1-KEN-05-A	*E. coli*	≤8/4	≤16/2	16	2	≤1	4	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	>2	>8	≤0.25	>4/76	≤0.25	8
S1-KEN-06-A	*E. coli*	≤8/4	≤16/2	16	2	≤1	>16	≤1	≤1	≤0.12	≤0.25	≤1	4	≤4	>2	8	≤0.25	>4/76	≤0.25	8
S1-KEN-06-B	*E. coli*	≤8/4	≤16/2	32	2	≤1	8	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	>2	8	≤0.25	>4/76	≤0.25	8
S1-KEN-07-A	*E. coli*	≤8/4	32/2	> 32	8	8	16	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	>2	8	≤0.25	>4/76	≤0.25	>16
S1-ZIM-01-A	*E. coli*	≤8/4	≤16/2	16	4	≤1	8	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	0.5	≤1	≤0.25	>4/76	≤0.25	>16
S1-ZIM-01-B	*E. coli*	≤8/4	≤16/2	32	2	≤1	8	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	0.5	≤1	1	>4/76	≤0.25	>16
S1-IVC-02-A	*E. coli*	≤8/4	≤16/2	8	≤1	≤1	8	≤1	≤1	≤0.12	≤0.25	≤1	8	≤4	>2	>8	≤0.25	2/38	≤0.25	>16
S1-ESP-02-A	*E. coli*	≤8/4	≤16/2	32	4	8	8	≤1	≤1	≤0.12	≤0.25	>8	>8	32	>2	8	0.5	>4/76	0.5	16
S1-ESP-03-A	*E. coli*	≤8/4	≤16/2	16	2	≤1	4	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	≤0.5/9.5	≤0.25	≤2
S1-SRB-02-B	*E. coli*	≤8/4	≤16/2	8	2	≤1	4	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	≤0.5/9.5	≤0.25	≤2
S1-HAI-01-A	*E. coli*	≤8/4	≤16/2	32	8	4	16	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	>2	>8	≤0.25	≤0.5/9.5	≤0.25	16
S1-VEN-01-A	*E. coli*	≤8/4	≤16/2	32	16	8	16	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	>2	>8	≤0.25	>4/76	≤0.25	>16
S1-ISR-02-A	*E. coli*	≤8/4	≤16/2	>32	4	8	>16	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	≤0.5/9.5	≤0.25	>16
S1-CHI-01-A	*E. coli*	≤8/4	≤16/2	4	≤1	≤1	≤1	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	>4/76	≤0.25	8
S1-CAM-02-A	*E. coli*	≤8/4	≤16/2	16	4	≤1	≤1	≤1	≤1	≤0.12	≤0.25	4	8	8	≤0.25	≤1	0.5	>4/76	≤0.25	8
S1-IND-01-A	*E. coli*	≤8/4	≤16/2	8	≤1	≤1	4	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	>4/76	≤0.25	≤2
S1-IND-01-B	*E. coli*	≤8/4	≤16/2	>32	8	8	16	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	>2	>8	≤0.25	>4/76	≤0.25	≤2
S1-IND-02-A	*E. coli*	≤8/4	≤16/2	>32	>16	>16	>16	≤1	≤1	≤0.12	≤0.25	>8	8	≤4	>2	>8	≤0.25	>4/76	≤0.25	>16
S1-THA-01-A	*E. coli*	≤8/4	≤16/2	8	≤1	≤1	≤1	≤1	≤1	≤0.12	≤0.25	>8	>8	≤4	>2	>8	≤0.25	≤0.5/9.5	≤0.25	8
S1-SRL-01-C	*E. coli*	>64/4	>128/2	>32	>16	≤1	>16	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	≤0.5/9.5	≤0.25	>16
S1-SRL-01-F	*E. coli*	≤8/4	128/2	2	8	≤1	≤1	≤1	≤1	≤0.12	≤0.25	≤1	≤1	≤4	≤0.25	≤1	≤0.25	≤0.5/9.5	≤0.25	>16
Clinical Breakpoints	S	≤8	≤8	≤1	≤1	≤1	≤1	≤2	≤2	≤1	≤0.5	≤2	≤2	≤8	≤0.25	≤0.5	≤0.5	≤2	≤2	N/A
	I																			
	R	>8	>16	>2	>4	>4	>4	>4	>8	>2	>0.5	>2	>2	>8	>0.5	>1	>0.5	>4	>2	N/A

P/T4; Piperacillin/tazobactam constant 4, TIM2; Ticarcillin/ clavulanic acid constant 2, FOT; Cefotaxime, TAZ; Ceftazidime, FEP; Cefepime, AZT; Aztreonam, IMI; Imipenem, MERO; Meropenem, DOR; Doripenem, ETP; Ertapenem, GEN; Gentamicin, TOB; Tobramycin, AMI; Amikacin, CIP; Ciprofloxacin, LEVO; Levofloxacin, TGC; Tigecycline, SXT; Trimethoprim/Sulfamethoxazole, COL; Colistin, DOX; Doxycycline.

Table 4: WGS and molecular characteristics from selected MDRE. Results are organized by similar MLSTs (n=20).

Strain ID	Sequence ID	MLST	Chromosomal ARGs	ARGs/ Plasmid Replicons (approximate size)	Other Plasmid replicons
S1-CHI-01-A[1]	*E. coli*	**ST69**	*bla*$_{TEM-1B}$, *aph(3'')-Ib, aph(6)-Id, dfrA7, mdf*(A), *sitABCD, sul1, sul2, tet*(A)	***bla*$_{CTX-M-15}$**, *qnrS1*/ **IncFII (72 kb)**	IncQ1, IncFIB(AP001918), IncFIA
S1-ESP-03-A[1]	*E. coli*	**ST69**	*mdf*(A), *sitABCD*	***bla*$_{CTX-M-15}$**, *qnrS1*/ **IncFII (72 kb)**	-
S1-SRB-02-B[1]	*E. coli*	**ST69**	*mdf*(A), *sitABCD*	***bla*$_{CTX-M-15}$**, *qnrS1*/ **IncFII (72 kb)**	-
S1-ISR-02-A	*E. coli*	**ST69**	*mdf*(A), *sitABCD*	***bla*$_{CTX-M-55}$**/ **IncI1-I(Alpha) (86 kb)** *dfrA14, erm*(B), *mdf*(A), *tet*(B)/ **IncFII(pRSB107), IncFII, IncFIB(AP001918), IncFIA (133 kb)**	ColRNAI*, Col156, Col(MG828), Col(BS512)
S1-IND-01-B	*E. coli*	**ST131**	***bla*$_{CTX-M-15}$**, *mdf*(A), *sitABCD*	*aadA5, dfrA17, erm(B), mph*(A), *sul1*/ **IncFII(pRSB107)** , **IncFIB(AP001918), IncFIA, Col156 (140 kb)**	-
S1-VEN-01-A	*E. coli*	**ST131**	***bla*$_{CTX-M-15}$**, *mdf*(A), *sitABCD*	*aadA5, dfrA1, erm(B), mph*(A), *sul* / **IncFII(pRSB107) , IncFIB(AP001918), IncFIA, Col156 (136 kb)** *aadA1, dfrA17, sul2, tet(B)* / **IncB/O/K/Z (110 kb)**	-
S1-KEN-05-A	*E. coli*	**ST131**	*mdf*(A), *sitABCD*	***bla*$_{CTX-M-27}$**, *aadA5, aph(3'')-Ib, aph(6)-Id, dfrA17, mph*(A), *sul1, sul2, tet*(A) / **IncFII(pRSB107) , IncFIB(AP001918), IncFIA, Col156 (118 kb)**	-
S1-THA-01-A	*E. coli*	**ST131**	***bla*$_{CTX-M-14}$**, *mdf*(A), *sitABCD*	*bla*$_{TEM-1B}$, *aac(3)-IId, aph(3'')-Ib, aph(6)-Id, sul2, tet*(A) / **IncFII(pRSB107) , IncFIB(AP001918), IncFIA, Col156 (100 kb)**	-
S1-SRL-01-C S1-SRL-01-F	*E. coli*	**ST349**	***bla*$_{DHA-1}$**, *mdf*(A), *mph*(A), *tet*(B), *qnrB4*, *sul*1	-	Col(pHAD28), Col(MG828)
S1-KEN-06-A S1-KEN-06-B	*E. coli*	**ST450**	***bla*$_{CTX-M-15}$**, *mdf*(A), *sitABCD*	*bla*$_{TEM\ family-like}$, *aph(3'')-Ib, aph(6)-Id, dfrA17, sul2, tet*(A)/ **IncFII(pRSB107), IncFIB(AP001918), IncFIA, Col156*, Col(BS512) (142 kb)**	-
S1-IND-01-A	*E. coli*	**ST616**	*mdf*(A)	***bla*$_{CTX-M-15}$**, *aadA5, dfrA17, qnrS1, sul1*/ **IncFIB(AP001918) (127 kb)**	Col8282, Col156, Col(pHAD28)
S1-CAM-02-A	*E. coli*	**ST1136**	*mdf*(A)	***bla*$_{CTX-M-27}$**, ***bla*$_{DHA-1}$**, *aph(3'')-Ib, aph(6)-Id, dfrA17, mph*(A), *qnrB4, sitABCD, sul1, sul2, tet*(A)/ **IncFII(Yp), IncFIC(FII), IncFIB(AP001918), IncFIA (145 kb)**	IncB/O/K/Z, ColRNAI
S1-ESP-02-A	*E. coli*	**ST1193**	***bla*$_{CTX-M-15}$**, ***bla*$_{OXA-1}$**, *aph(3'')-Ib, aph(6)-Id, dfrA17, sul2, tet*(B)	*aac(3)-IIa, aac(6')-Ib-cr, mdf* (A), *sitABCD*/ **IncQ1, IncFIB(AP001918), IncFIA, Col156 (92 kb)**	Col8282, Col156*, Col(MG828), Col(BS512)
S1-KEN-07-A	*E. coli*	**ST1193**	***bla*$_{CTX-M-15}$**, *mdf*(A), *sitABCD*	*bla*$_{TEM-1B}$, *aph(3'')-Ib, aph(6)-Id, dfrA17, mph*(A), *sul2, tet*(B)/ **IncQ1, IncFIB(AP001918), IncFIA, Col156 (102 kb)**	ColRNAI*, Col8282, Col156, Col(BS512)
S1-HAI-01-A	*E. coli*	**ST4988**	***bla*$_{CTX-M-15}$**, *mdf*(A)	*aph(3'')-Ib, aph(6)-Id, sul2, tet*(A)/ **Col(pHAD28) (8 kb)**	IncX4, ColRNAI*, Col156, Col(MG828), Col(BS512)
S1-KEN-03-A[2] S1-KEN-03-C[2] S1-KEN-04-A[2]	*E. coli*	**ST5614**	*mdf*(A)	***bla*$_{CTX-M-15}$**, *aadA5, aph(3'')-Ib, aph(6)-Id, dfrA17, mph*(A), *qnrS1, sul1, sul2, tet*(A)/ **IncB/O/K/Z (126 kb)**	ColRNAI, Col(MP18)

[1] Plasmid sequences were used to construct **Figure 4.1**. [2] Plasmid sequences were used to construct **Figure 4.2**. (* = 2 or more).

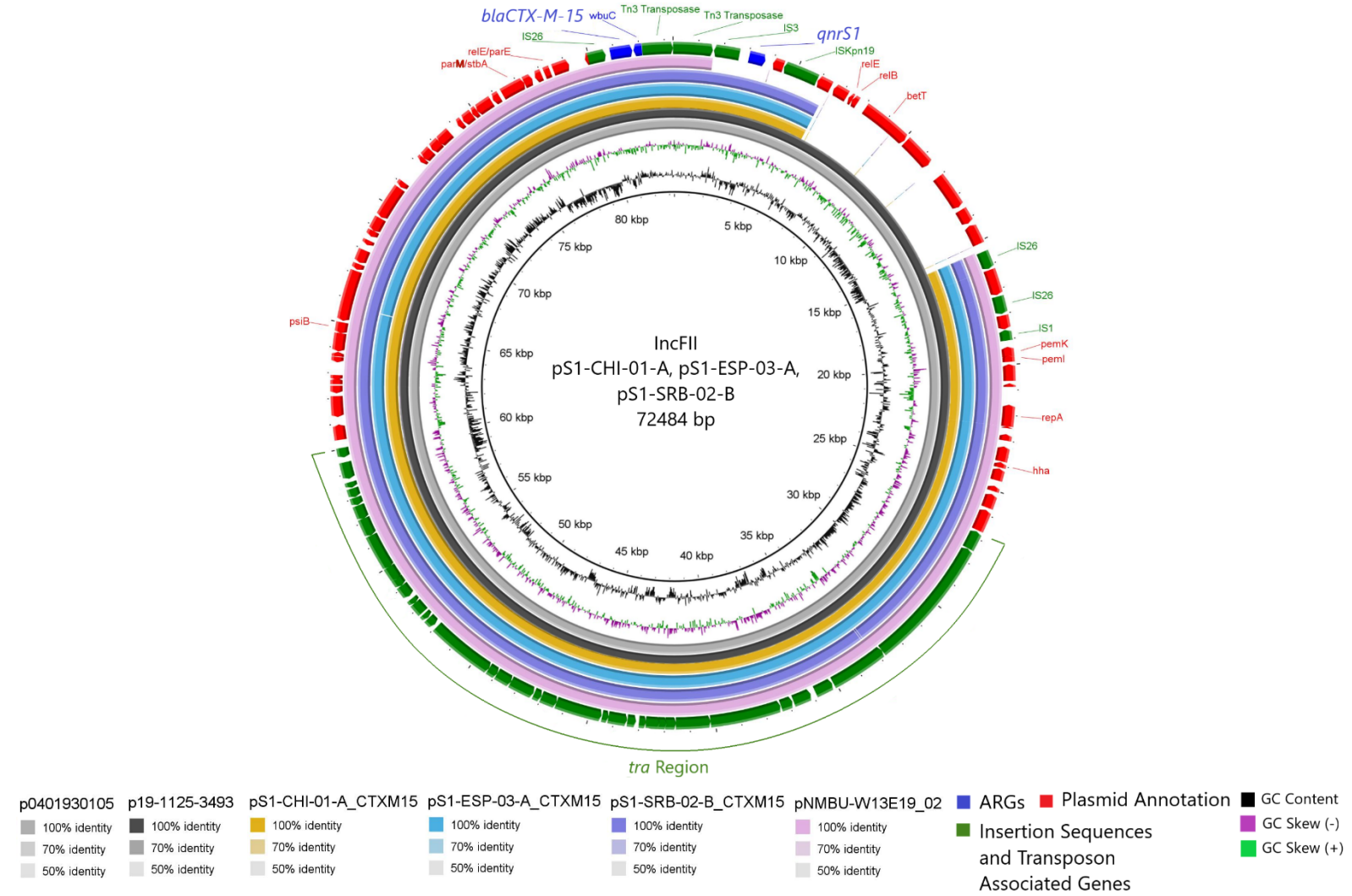

Figure 4.1: BLASTn comparison of IncFII plasmids from study MDRE strains: S1-CHI-01-A, S1-ESP-03-A, and S1-SRB-02-B carrying *bla*$_{\text{CTX-M-15}}$ against an *E. coli* reference pNMBU-W13E19 plasmid (GenBank accession number NZ_CP043408.1). Two *bla*$_{\text{CTX-M-15}}$-containing *Shigella sonnei* plasmids p0401930105 and p19.1125.3493 (GenBank accession numbers: CP049172.1 and CP049170.1) were also included to show sequence similarity to study plasmid samples. Rings were generated using the BRIG software as described above.

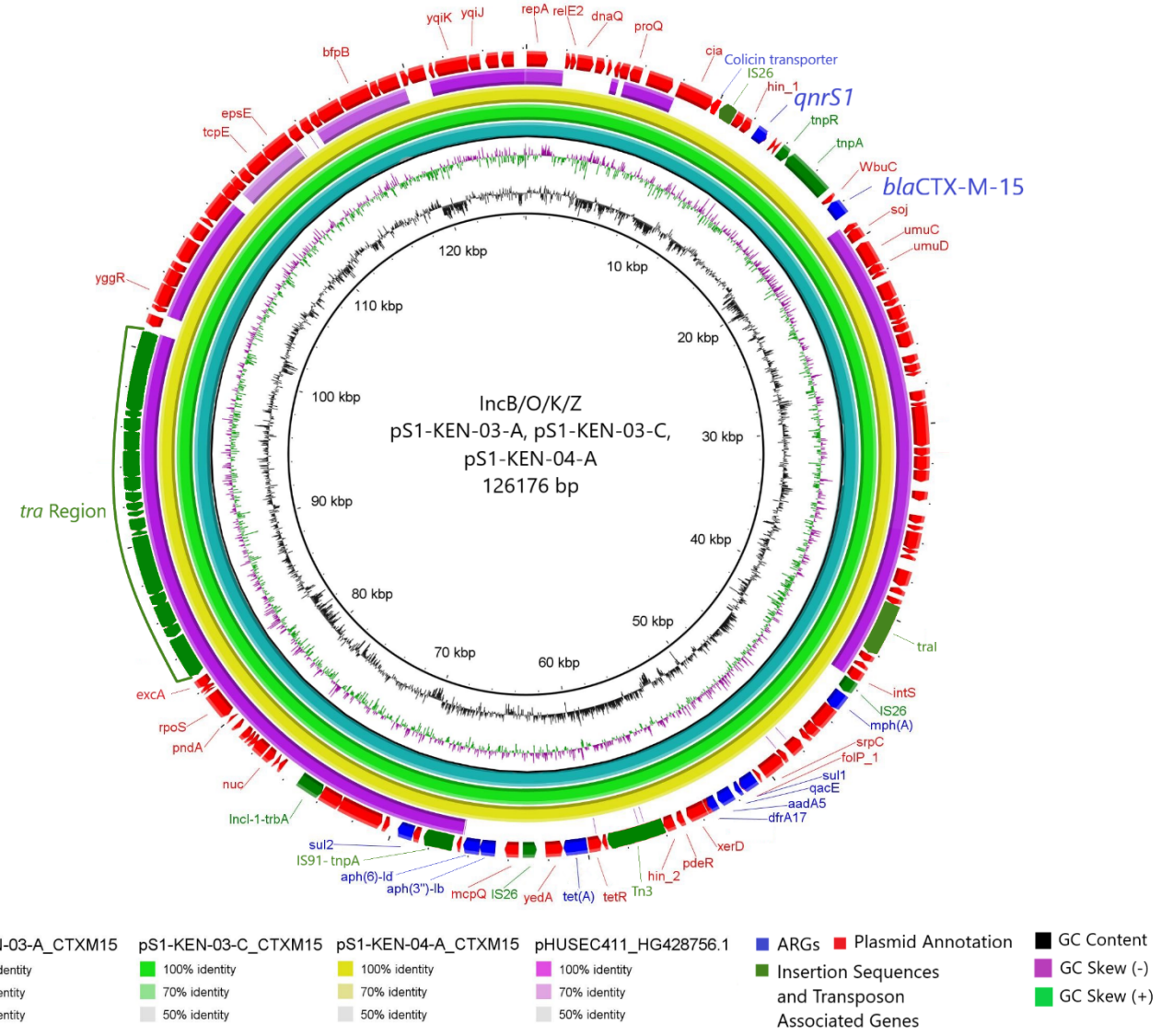

Figure 4.2: BLASTn comparison of IncB/O/K/Z plasmids from study MDRE strains: S1-KEN-03-A, S1-KEN-03-C, and S1-KEN-04-A carrying *bla*$_{\text{CTX-M-15}}$. *E. coli* pHUSEC411-like plasmid was used for ARG comparison (GenBank accession number HG428756.1). Rings were generated using the BRIG software as described above.

The Hidden Gut Resistome is a Reservoir for ARGs

Metagenomic resistome characterization was performed on a selection of stools from participants with different MDRE colonization status (8 MDRE-colonized, and 12 non-MDRE-colonized). Metagenome assemblies were prepared from Illumina reads as described above (see materials and methods). We identified various ARGs that have been previously characterized in putative resistance to many antibiotic classes (**Table 5**). In **Table 5**, ARGs that were characterized in putative resistance profiles to the same antibiotic classes were grouped together for ease of interpretation (e.g., *aac*(6'), *ant*(6)-Ia, *aph*(3')-III, *aph*(2''), *npm*A for aminoglycoside resistance were included as: *aac-ant-aph-npm*).

Generally, we found no clear difference in the resistome profile between MDRE-colonized and non-MDRE-colonized participants. This can clearly be seen in that we discovered genes involved in resistance to β-lactam antibiotics in 19/20 screened study participants regardless of their MDRE-colonization status. Furthermore, we identified genes involved in aminoglycoside (AMG), tetracycline (TET), and macrolides-lincosamides-streptogramins (MLS) resistance in all 20/20 of the screened study participants, also regardless of their MDRE-colonization status. Less commonly, we discovered genes involved in chloramphenicol (CHL) resistance in 6/8 MDRE-colonized participants, and in 9/12 non-MDRE-colonized participants. Surprisingly, we only identified genes involved in fluoroquinolone (FQ) resistance in two MDRE-colonized study participants, and in two non-MDRE-colonized study participants. Furthermore, we found genes involved in disrupting folate metabolism (e.g., *dfr*A17, *sul*1 and *sul*2) in four MDRE-colonized participants, and in four non-MDRE-colonized participants [32, 33]. In two MDRE-colonized and one non-MDRE-colonized participant we found *nim* genes involved in resistance to nitroimidazole (NIM) antibiotics such as metronidazole [34].

Interestingly, we identified three β-lactamase genes: $bla_{\text{OXA-347}}$, $bla_{\text{OXA-212}}$, and $bla_{\text{ACI-1}}$ which we had not identified in the isolated MDRE strains. From the participants who were colonized with MDRE, we found that only S1-THA-01 and S1-ESP-03 carried identifiable *bla* genes in their resistome. Significantly, participant S1-ESP-03 was the only characterized participant in whom we identified $bla_{\text{CTX-M-15}}$ both in an isolated MDR *E. coli* and in their metagenomic reads. This participant was also the only participant in which we identified more than one *bla* gene ($bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-347}}$, and $bla_{\text{ACI-1}}$).

In participants who were not colonized with MDRE we identified various *bla* genes including the $bla_{\text{TEM-1B}}$ gene. $bla_{\text{TEM-1B}}$ was identified in many of the isolated MDRE strains, but was only identified through metagenomic screening in participant S1-TAN-01. Moreover, we identified the rare *van*G2XY gene in the resistome of two non-MDRE-colonized participants (S1-ISR-01 and S1-GER-05). The *van*G2XY gene is commonly found in Gram-positive *Enterococcus* species which colonize the gut microflora and has been characterized in particularly dangerous nosocomial infections and transfer into resistant *Staphylococcus aureus* strains [35, 36].

Table 5: Putative resistance (i.e., ARGs) to several antibiotic classes detected by using the metagenomic approach in stool samples from study participants (n=20). Fill (yellow) indicates that ARGs with putative resistance were identified. No fill indicates no relevant ARGs were identified.

Participant ID	MDRE Colonization Status	*bla* Genes Detected	Rare Genes Detected	Putative Resistance to Antibiotic Classes (by ARG families)										
				PCN	3GC	4GC	AMG	FQ	TET	GP	AF	CHL	NIM	MLS
				cfx, cep	*cfx, cep*	*cfx, cep*	*aac, ant, aph, npm*	*qnr, mdf*	*tet*	*van*	*dfr, sul*	*cat, cfr*	*nim*	*erm, msr, mef, lnu, ere*
S1-KEN-04	+ MDR *E. coli*	-	-											
S1-CHI-01	+ MDR *E. coli*	-	-											
S1-IND-01	+ MDR *E. coli*	-	-											
S1-SRL-01	+ MDR *E. coli*	-	-											
S1-ISR-02	+ MDR *E. coli*	-	-											
S1-THA-01	+ MDR *E. coli*	***bla*$_{OXA-347}$**	-											
S1-ESP-02	+ MDR *E. coli*	-	-											
S1-ESP-03	+ MDR *E. coli*	***bla*$_{CTX-M-15}$, *bla*$_{OXA-347}$, *bla*$_{ACI-1}$**	-											
S1-TAN-01	Negative	***bla*$_{TEM-1B}$**	-											
S1-TAN-02	Negative	-	-											
S1-ISR-01	Negative	**-**	***Van*G2XY**											
S1-THA-02	Negative	***bla*$_{OXA-212}$**	-											
S1-ESP-01	Negative	-	-											
S1-GBR-01	Negative	***bla*$_{OXA-347}$**	-											
S1-GER-01	Negative	***bla*$_{ACI-1}$**	-											
S1-GER-02	Negative	-	-											
S1-GER-03	Negative	-	-											
S1-GER-04	Negative	***bla*$_{ACI-1}$**	-											
S1-GER-05	Negative	***bla*$_{OXA-347}$**	***Van*G2XY**											
S1-GER-06	Negative	-	-											

PCN, Penicillins; 3GC, 3rd Generation Cephalosporins; 4GC, 4th Generation Cephalosporins; AMG, Aminoglycosides; FQ, Fluoroquinolones; TET, Tetracyclines; GPs, Glycopeptides; AFs, Antifolates; CHL, Chloramphenicol; NIM, Nitroimidazole; MLS, Macrolides-Lincosamide-Streptogramins [11, 28, 33-35, 37-40].

Study Participants in African and Asian Regions are at Highest Risk for MDRE Colonization

From 57 enriched stool samples, we determined that study participants who were stationed in countries in the African region were at significant risk for developing MDRE colonization (OR 3.8; 95% CI 0.81-17.92) compared to participants stationed in Europe. Similarly, study participants stationed in the Asian geographical region showed the highest risk of MDRE colonization (OR 6.0; 95% CI 1.20-30.01) when compared to participants stationed in Europe (**Table 2**, **Figure 3** and **Figure 5**).

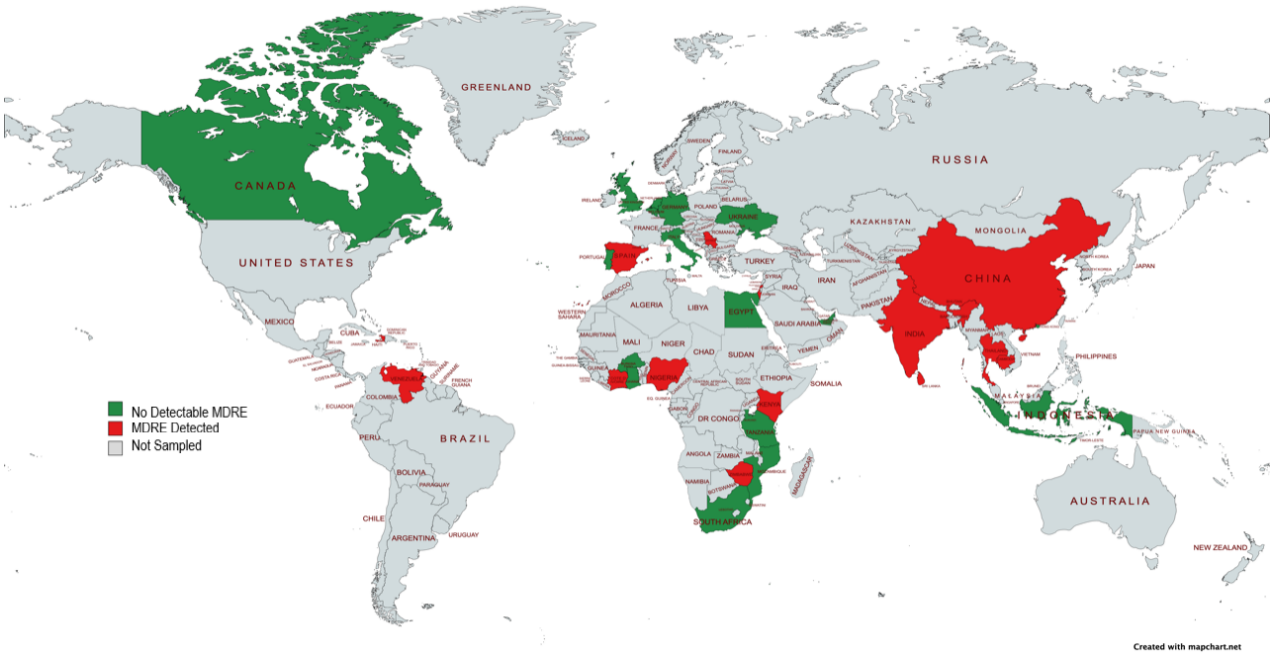

Figure 5: Geographical locations of Swiss expat volunteers who have sent in stool samples so far. Countries from which at least one screened stool sample was positive for MDRE are filled in red. Countries from which all screened stool samples were negative for MDRE are filled in green.

Identified Risk Factors for Developing MDRE Colonization

Study participant's responses to the epidemiological questionnaire were used to classify their demographics, health history, lifestyle, and diet. Odds ratios were then used to evaluate potential risk factors for MDRE colonization.

We found that males were at higher risk than females for being colonized with MDRE (OR 1.38; 95% CI 0.48-3.89). We also found that participants who had pets (OR 1.61; 95% CI 0.48-5.48) were at higher risk for colonization with MDRE. Furthermore, we found that the 36-50-year-old age group (OR 2.72; 95% CI 0.82-9.04), those who used antibiotics within the last two years (OR 2.58; 95% CI 0.74-8.94), and those who had diarrhea within the last three months (OR 2.41; 95% CI 0.78-7.46) were all at much higher risk for colonization with MDRE (**Table 6**). Furthermore, we identified that participants who traveled to Africa (OR 3.38; 95% CI 1.00-11.80) and those who traveled to Asia (OR 2.13; 95% CI 0.50-9.04) for different reasons (e.g., vacation or mission) were at very high risk of developing MDRE colonization.

Travel to the Americas, eating outside of the place of residence, and consuming alcohol were not associated as risk factors for colonization with MDRE. Those who were regular milk and tap water consumers, as well as those participants engaged in sports (mostly outdoor sports such as hiking, walking, running, and biking) were associated with being at a lower risk for developing colonization with MDRE (**Table 6**). We were unable to associate the level of risk

for those with previous hospitalizations and those who are cheese consumers due to insufficient positive responses to these questions. However, other studies have found that previous hospitalizations were risk factors for developing MDRE colonization [16]. It is possible we will achieve a similar result as the sample size of the present study grows.

Table 6: Risk factors for developing colonization with MDRE. Odds were interpreted as OR<1; decreased risk, OR=1 no association, OR>1 increased risk. 36-50-year-olds were compared against 18-35-year-olds and >50-year-olds. Participants were characterized as alcohol, milk, cheese, or tap water consumers if they had at least one serving per week.

Risk Factor	Odds Ratio	95% Confidence Interval
Stationed in Asia	**6.0**	1.20-30.01
Stationed in Africa	**3.8**	0.81-17.92
Travel to Africa	**3.38**	1.00-11.80
36-50-Year-olds	**2.72**	0.82-9.04
Use of Antibiotics in Last 2 years	**2.58**	0.74-8.94
Diarrhea in Last 3 months	**2.41**	0.78-7.46
Travel to Asia	**2.13**	0.50-9.04
Having Pets	**1.61**	0.48-5.48
Sex (Males)	**1.38**	0.48-3.89
Drinking Alcohol	**1.06**	0.36-3.13
Eating out often (>4x/Week)	**1.05**	0.34-3.24
Travel to the Americas	**1.02**	0.22-4.84
Hospitalization	**0.92**	0.08-10.83
Milk consumers	**0.89**	0.27-2.9
Sports Engagement	**0.74**	0.25-2.24
>50-Year-olds	**0.62**	0.56-1.86
Cheese consumers	**0.53**	0.03-8.92
Tap water consumers	**0.44**	0.15-1.34
18-35-Year-olds	**0.35**	0.07-1.79

Discussion:

It has been established that gut colonization with MDRE can vary significantly due to a variety of factors [5, 8-10, 12, 13, 16, 17]. Recent studies have determined significant risk factors for MDRE colonization include international travel to high MDRE prevalence areas, antibiotic use in the previous 3-12 months, inflammatory bowel disease, and diarrhea [5, 8-10, 12, 16, 17]. Unfortunately, many of these studies only follow participants for a short time and study populations are restricted to travelers with short stays in high MDRE prevalence countries [8-10, 12, 17]. Moreover, studies following participants from countries with low

MDRE prevalence who live in high prevalence countries for extended periods of time (at least three months) have never been done before at this scale. Studying this unexplored population may have significant implications into the One Health perspective of MDRE throughout the world.

In order to address these concerns, we collected stools from 57 Swiss expats stationed throughout the world to better understand the characteristics, hidden resistome, and risk factors for MDRE colonization during extended stays in high prevalence countries.

Prevalence of Colonization with MDRE: geographic differences

We recorded that 33% of our study population was colonized with MDR *E. coli* (**Table 2** and **Figure 3**). Furthermore, we found a higher proportion of colonized study participants were stationed in Africa and Asia as opposed to Europe and the Americas (**Figures 3 and 5**). Similar results have been reported in previous studies that show the African and Asian regions to be high MDRE prevalence areas where travelers are often colonized [9, 10, 18-20, 22]. High proportions of MDRE colonization have never been represented in long-term foreign residents in endemic regions until now. Our data represent that extended periods of stay in MDRE-endemic countries parallel the MDRE colonization dynamics seen in international travelers. Therefore, extended periods of stay in high prevalence countries should be treated as a significant risk factor for MDRE colonization. We speculate that as individuals spend more time in high prevalence countries their risk for developing MDRE colonization should also increase. This will be assessed as the sample size of this study grows and multiple screenings are performed on our study participants.

Global Dissemination of ESBLs

A study performed in 2016 by Karanika *et al*. speculated that around 14% of the world population was colonized with ESBL-producing *Enterobacterales* [20]. Furthermore, the study claimed that this colonization rate was increasing yearly by 5%, but showed steeper increases in regions such as Africa and Southeast Asia thus shaping the pandemic of MDROs [19, 20]. More recent estimates suggest that at least 16.5% of healthy individuals are colonized with an ESBL-producing *E.* coli [41]. We demonstrated that all of the MDRE that we identified had an ESBL-producing phenotype by MIC testing (33% of our study sample; **Table 2** and **Table 3**). Although we identified more ESBL-positive strains from the African and Asian geographical regions, we demonstrated that these MDR pathogens were in every geographical region that we sampled. This evidence supports the One Health concern of the pandemic of MDROs and suggests that long-term international residents may greatly contribute to the global expansion of these life-threatening bacteria [2]. In previous studies, local residents from Tanzania and Laos (high-prevalence countries) showed alarming high levels of colonization with MDRE (91.5% and 70%, respectively) [14, 42]. Our concern is that through extended periods of stay in these countries, Swiss expatriates may begin to reflect this colonization prevalence and return to Switzerland carrying resistant isolates and propagate them in the community and hospital setting. This phenomenon may also be reflected in other European countries with low prevalence of MDRE colonization.

Moreover, the higher proportion of MDRE that we identified as compared to previous studies (16.5% *vs*. 33%) may suggest that antibiotic enrichment is an essential step in identifying the presence of these pathogens in healthy people (**Table 2**) [41]. The importance of using antibiotic enrichments to detect MDRE has also been described in previous studies [17]. Since the sample size of the present study is still small, the prevalence of volunteers colonized with MDRE may not reflect the true epidemiology. Overall, future studies that survey the global prevalence of MDRE and other MDRO should be performed with an

antibiotic enrichment protocol to ensure that the gut colonization status of participants is more accurately reported.

WGS of MDRE

ESBLs of CTX-M type in humans were first described in 1989 in a Munich isolate that was capable of hydrolyzing cefotaxime [11]. Among the most significant *bla*$_{\text{CTX-M}}$ genes there is the *bla*$_{\text{CTX-M-15}}$ which is spreading in both hospital and community settings and showing a great clinically relevance [43]. *bla*$_{\text{CTX-M-15}}$ is found both chromosomally and in plasmids which speaks both to its stability in the genome of many MDROs and to its importance in the pandemic of MDROs [11]. In fact, the majority of our study isolates contained *bla*$_{\text{CTX-M}}$ genes, mainly *bla*$_{\text{CTX-M-15}}$. We even identified four isolates which belonged to the pandemic ST131 clone that carried either *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-27}}$, or *bla*$_{\text{CTX-M-14}}$ with variability in chromosomal or plasmid carriage of the genes [27]. This finding likely indicates that different acquisition events of *bla*$_{\text{CTX-M}}$ genes are occurring in isolates of similar STs which adds to the complexity of dealing with these MDRE.

Interestingly, isolate S1-CAM-02-A - which shows resistance to ESCs - had a plasmid-located *bla*$_{\text{CTX-M-27}}$ ESBL gene and a *bla*$_{\text{DHA-1}}$ plasmidic AmpC gene (**Table 4**) [11]. In this particular instance, the isolate was still sensitive to the β-lactamase inhibitor combinations, to the 4$^{\text{th}}$-generation cephalosporin cefepime, and to the monobactams [11, 25, 44].

We characterized two interesting scenarios of plasmid carriage of the *bla*$_{\text{CTX-M-15}}$ gene. In one scenario, the *bla*$_{\text{CTX-M-15}}$ was located downstream of an IS*26* element in a seemingly identical IncFII plasmid in three study isolates found in people who did not share personal or professional connection (S1-CHI-01-A, S1-ESP-03-A, S1-SRB-02-B; **Table 4** and **Figure 4.1**). This scenario illustrates the extent of global dissemination of plasmidic *bla*$_{\text{CTX-M-15}}$. In an alternate scenario, we identified the *bla*$_{\text{CTX-M-15}}$ downstream of an IS*26* element in an IncB/O/K/Z plasmid in three isolates from two related participants (S1-KEN-03-A, S1-KEN-03-C, and S1-KEN-04-A; **Figure 4.2**). This scenario illustrates the relevance of plasmidic *bla*$_{\text{CTX-M-15}}$ in the community setting, especially in those individuals who share a household [43]. As the sample size of the study grows, we will likely see more instances of both of these scenarios.

Furthermore, in many of our MDRE isolates we identified ARGs to other antibiotic classes such as fluoroquinolones and aminoglycosides which may have an additive effect on the overall resistance phenotype of these isolates [29, 30]. Interestingly, however, with our WGS approach we identified ARGs that did not agree with the results from our phenotypic analyses (MIC). For example, in isolate S1-CHI-01-A we described a plasmid which contained *bla*$_{\text{CTX-M-15}}$ and *qnr*S1 genes. This isolate should not be susceptible to ESCs and to fluoroquinolone antibiotics. However, we see complete susceptibility to the fluoroquinolone antibiotics and a relatively low grade ESBL phenotype when compared to the other two strains which carry the same plasmid. Nevertheless, this particular isolate is still more resistant than the wild-type isolate (mic.eucast.org) which indicates that there is some expression of the resistance profile. Perhaps, this isolate does not express the resistance phenotype as effectively in S1-CHI-01-A. Furthermore, the promoters and IS elements in the S1-CHI-01-A may not be actively inducing transcription of the ARGs, therefore leading to the increased susceptibility.

Hidden Gut Resistome as an ARG Reservoir

Many metagenomic studies have been performed to characterize the unculturable bacteria that make up the complex gut microbiome environment [45-47]. With an estimated 2-4 million genes in this environment, it is clear that this is a field that requires extensive study and attention [47]. Currently, there is a large gap in knowledge of the metagenome of

individuals from low MDRE prevalence countries who reside long-term in high prevalence countries.

We were surprised to find that the hidden gut resistome was very similar in participants who were colonized by MDRE and in those who were not colonized with MDRE. This may be due to the previously observed phenomenon that metagenomics approaches are not sensitive enough to detect ARGs that are present in low abundance in the human gut [45]. Many clinical laboratories perform ARG screenings directly on stool samples and may be missing a large proportion of significant ARGs that are present in low abundance [46]. In this context, metagenomics analyses are powerful tools for determining the hidden gut resistome of difficult-to-culture organisms, but may not be suitable for diagnostic purposes in the clinical laboratory. Furthermore, in many of the MDRE-colonized participants that we tested we were unable to identify significant ARGs such as *bla*$_{\text{CTX-M-15}}$ in the metagenomic reads. In fact, we were only able to do this in one participant (S1-ESP-03; **Table 5**) indicating that this individual likely has high abundance of *bla*$_{\text{CTX-M-15}}$ in their gut resistome. This adds credibility to our antibiotic enrichment approach when screening for MDROs. Seeing that many significant ARGs (e.g., *bla*$_{\text{DHA-1}}$, *bla*$_{\text{CTX-M-27}}$, and *bla*$_{\text{CTX-M-14}}$, etc.) were identifiable only after the antibiotic enrichment, this protocol should be implemented in all diagnostic labs that aim to characterize the full extent of MDRE colonization in patient samples. It is important to note that our study was performed on healthy participants who are not currently suffering from pathogenic intestinal infections. In hospitalized patients with pathogenic MDRO infections, metagenomic analyses of the hidden gut resistome may identify more significant ARGs involved in intestinal pathogenesis and should be explored in further studies.

Nevertheless, we identified a large set of ARGs in the hidden gut resistome of our study participants. These genes ranged from resistance genes with putative resistance to extended-spectrum cephalosporins, to macrolides-lincosamide-streptogramins, and to many genes for tetracycline resistance [32-34]. In the gut microbiome environment, commensal bacteria and pathogenic bacteria can share ARGs and MGE even across phyla [48]. This is alarming in the context of rare ARGs such as *van*G2XY, which we identified in the resistome of two individuals, and has been characterized in Gram-positive species of *Enterococcus* and in pathogenic *Staphylococcus aureus* strains [35, 36]. Our data highlight the importance of antibiotic enrichment in detecting MDRE and further reveal the resistome of the gut microbiome environment is an important reservoir for a wide range of ARGs.

Risk Factors for MDRE Colonization

Many studies have been performed to better understand the risk factors for MDRE colonization. A recent study by Collignon *et al*. revealed that certain anthropological and socioeconomic factors such as quality of governance and access to clean drinking water have an influence on the level of antibiotic resistance present in communities [13]. Furthermore, travel to countries where these measures are lacking and MDRE are prevalent has been established as a significant risk factor for MDRE colonization [5, 8-10, 12, 13, 16, 18]. Our present study also identified international travel to high MDRE prevalence regions (Africa and Asia) to be a significant risk factor for colonization, whereas we did not determine travel to low prevalence regions (Americas and Europe) to be a risk factor for colonization (**Table 6**).

In our study population, we found that males were at greater risk for MDRE colonization than females (**Table 6**). This could be due to males being more biologically susceptible to MDRE colonization than females, or that males engage in riskier activities for MDRE colonization (such as international travel) as opposed to females. This can be similarly said for the 36-50-year-old age group as opposed to the 18-35 and >50-year-old age groups. Perhaps the 36-50-year-olds are biologically more susceptible to MDRE colonization or they

engage in riskier activities for MDRE colonization. These hypotheses should be explored in future studies and as the sample size of this study increases.

Our data also indicate that having pets increases the risk of MDRE colonization (**Table 6**). A recent study following the intestinal carriage of ESC *Enterobacterales* by Pires *et al.* showed that pets and owners within the same household were colonized with the same ESC *E. coli* [49]. Similarly, a study performed by Endimiani *et al*. demonstrated that Swiss veterinary professionals were carrying strikingly similar carbapenemase-producing *Enterobacterales* strains as dogs and cats within the same clinics [50]. Our data suggest that pets may be an important source of human infection with MDRE and should continue to be studied as a relevant risk factor.

Antibiotic use within the last two years and diarrhea within the last three months were both determined to be significant risk factors for MDRE colonization (**Table 6**). However, it is difficult to conclude which of these factors precedes the others. Whether MDRE colonization causes diarrhea and antibiotic use, or whether antibiotic use and diarrhea are precursors for colonization with MDRE is unknown. These measures have been discussed in previous studies and nevertheless provide a good indication for colonization with MDRE [16].

Lastly, it should be noted that many of our confidence intervals span 1.0 thereby reducing the statistical power of our results. However, the sample size of the study remains quite small, and is projected to include at least 250 participants. Therefore, this current issue should be resolved as our sample size grows to the projected figure and we are confident this study will provide valuable information for ameliorating the global health problem of antibiotic resistance.

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