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Article

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Gut resistome presents a unique biome signature in chronic multi-symptom illness patients and links persistent pro-inflammatory phenotype in a mouse model reversible by fecal microbiota transfer

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Abstract:

Chronic multi-symptom illness (CMI) affects a subsection of elderly and war veterans and is associated with systemic inflammation, chronic fatigue, pain and neuroinflammation. We showed previously that an altered gut microbiome-inflammation axis aids to the symptom reporting and persistence. Here, a mouse model of CMI and a group of Gulf War veterans' with CMI showed the presence of an altered host resistome, a signature of antibiotic resistance genes within the microbiome. Results showed that antibiotic resistance genes were significantly altered in the CMI group in both mice and GW veterans when compared to the control. Fecal samples from GW veterans with persistent CMI showed a significant increase of resistance to a wide class of antibiotics and exhibited an array of mobile genetic elements distinct than normal healthy controls. Strikingly, the altered resistome and gene signature were correlated with mouse serum IL6 levels. Altered resistome in mice also correlated strongly with intestinal inflammation, decreased synaptic plasticity that was reversible with fecal microbiota transplant (FMT), a tool to restore a healthy biome. The results indicate an emerging linkage of the gut resistome and CMI and might be significant in understanding the risks to treating hospital acquired infections in this population.

Introduction
Antibiotic resistance has emerged as a threat to public health on the local and global scale [1], [2]. Resistance to antibiotics is conferred on bacteria by antibiotic resistance genes (ARGs). All of the ARGs collectively form a resistome, and usually are carried by the opportunistic pathogens, humans may encounter thus increasing the risk of acquired infection that are difficult to treat with currently approved antibiotics[3], [4], [5], [6]. The evolution of drug resistance in such pathogens is driven by chromosomal mutation and the acquisition of ARGs. Since most of the ARGs are linked to mobile genetic elements (MGEs), they can transfer easily through horizontal gene transfer (HGT) among different clones, taxa and habitats [7], [8], [9], [9], [10].

The human gut harbors multiple commensal microorganisms and forms a good reservoir of ARGs. The gut environment predisposes ARG transfer, sometimes leading to emergence and spread of specific bacterial clones carrying genes of resistance and/or virulence. The most frequently reported genes are those directed against tetracycline, β-lactams, aminoglycosides and glycopeptides. Tetracycline and glycopeptide resistant genes are most common in fecal samples of human [4], [11], [12], [13]. On the other side, human microbiota is also influenced by environmental factors that can contribute to alteration of microbial composition and affect colonization resistance to pathogens [14]. A report from Sun et al., shows that temporary changes in living environment can alter the human gut microbiota and resistome [12]. Another similar study by Parnanen et al. suggested that host also can alter the microbiome and the gut resistome by transmission of ARGs from mother to infant via breast milk [15].

Numerous studies have reported that elderly and war veterans often suffer from multiple syndromes with inflammatory phenotypes and are more susceptible to resistance against
a large number of antibiotics [16], [17], [18]. Veterans have been reported to be resistant
to amoxicillin, β-lactams, fluoroquinolones, methicillin and most importantly the
carbapenem group of antibiotics [19], [20]. In our present study, we aimed to analyze the
ARG and mobile genetic element (MGE) patterns in chronic multisymptom illness (CMI)
in a GW mouse model as well as in a cohort of GW veterans. GW illness (GWI) is a
chronic multisymptomatic illness (CMI) or condition reported by the veterans returning
from Operation Desert Storm/Desert Shield in 1991. Symptoms reported by the veterans
include fatigue, headache, cognitive dysfunction, musculoskeletal pain, respiratory and
gastrointestinal dysfunctions more often characterized by a persistent systemic
inflammation with higher IL6, TNF-R1 and IL1β blood levels. These symptoms have been
linked to chemical exposures experienced during the war. [21, 22], [23].

Several studies in preclinical mouse models of CMI related to GW have reported that
exposure to chemicals such as insecticides and anti-nerve gas resulted in gut microbial
dysbiosis. There has been a decrease in the relative abundance of several beneficial
bacteria. Recently, a study in GW-CMI veterans also reported similar alteration of gut
microbiome [24], [25], [26]. With reported studies on microbial dysbiosis patterns in the
preclinical animal models as well as the GW-CMI veteran well established, we
hypothesized that exposure to environmental pesticides and pyridostigmine bromide
may also lead to an alteration of ARGs and MGE expression, an important constituent of
the gut resistome. In the present study we used both GWI veteran stool samples and
fecal pellets from a GWI persistence mouse model mimicking the present day veteran
health to study the alteration in gut resistome. We also aimed to study the possible
associations between gut resistome and CMI proinflammatory pathology and
mechanistically linked the altered resistome with a proinflammatory phenotype by using a fecal microbiota transfer in mouse models.

**Patients/Materials and methods**

Permethrin (Per), Pyridostigmine bromide (PB) were purchased from Sigma-Aldrich. Primary antibodies anti-interleukin-1β(IL-1β), anti-brain derived neurotrophic factor (BDNF) were purchased from Santacruz Biotechnology (Dallas, TX, USA). Species specific biotinylated secondary antibodies and streptavidin-HRP (Vectastain ABC Kit) were purchased from Vector laboratories (Burligame, CA, USA). All other chemicals used in the present study were purchased from Sigma unless specified. Animal tissues were sent for paraffin embedding and sectioning to AML Laboratories (Baltimore, MD, USA). Fecal samples from experimental mice groups and GWI veterans were sent to COSMOSID (Rockville, MD, USA) for whole genome sequencing.

**Animals**

C57BL/6J wild type mice of 10 weeks age were purchased from Jackson Laboratories (BarHarbor, ME, USA). The mice were maintained in accordance with local IACUC standards and National Institute of Health guidelines for human care and use of laboratory animals. All animal experimental procedures were approved by University of South Carolina at Columbia, SC. All the mice had ad libitum access to food and water and were housed at 22-24°C with 12h light/12h dark cycles. The mice were sacrificed after the animal experiments. Organs including frontal cortex and distal part of small intestine were collected after dissecting the mice and fixed in Bouin’s solution and 10% neutral buffered formaldehyde respectively. Serum was collected from fresh blood of mice by performing
cardiac puncture after anesthesia. The fecal pellets were collected from colon and it was stored at -80°C for whole-genome sequencing.

**Mouse model of CMI related to Gulf War exposures.**

After one week of acclimatization, the mice were randomly distributed into three groups. The first group received vehicle (0.6% dimethyl sulfoxide) for two weeks and were denoted Control (n=6). The second and third mice groups denoted GWI(n=6) and GWI_FMT(n=6) were treated with Per (200mg/kg body dissolved in DMSO and phosphate buffer saline(PBS) and PB (2mg/kg dissolved in PBS) by oral gavage tri-weekly for 15 days. After the 2 weeks of GW chemical exposure, GWI group mice were allowed to persist for 20 weeks. Fecal microbiota transplant was administered in GWI_FMT after GW chemical exposure. 100mg of fecal pellets were collected from healthy C57BL/6J mice of same age group as the GWI_FMT mice. The pellets were homogenized in 1ml of PBS and centrifuged at 3000g for 5 min. 100μl of supernatant was dosed in each mice on alternate days of a week for 20 weeks [27].

**Human Subjects: GW veterans with CMI and controls**

The Boston Gulf War Illness Consortium (GWIC) performs preclinical and clinical studies in order to understand the pathophysiology behind the complex symptoms in GW veterans in order to aid in designing of possible therapeutic strategies. Veterans were included as participants in GWIC studies on the basis of requirement that they had to be deployed in the Gulf War i.e. from August 1990 to July 1991. The GWIC used the Kansas GWI criteria as the case definition which requires the veterans to have symptoms in 3 out of 6 broadly defined group of symptoms (neurological, pain, gastrointestinal, skin,
respiratory, fatigue) to meet criteria of CMI in GW veterans known as Gulf War Illness (GWI)\cite{26},\cite{28}. GW veterans who do not meet Kansas criteria are deemed the control group.

The veterans who participated in the GWIC, underwent multiple tests including neuropsychological assessments, health surveys, biological specimen collection and brain imaging \cite{22}. The present study was conducted as a GWIC call back study in which we aim to reassess 150 of the GWIC participants. For this study, data from the first 15 recruited subjects from the microbiome call back study has been analyzed. The recruitment of participants was via telephone on completion of GWIC study protocol. After filling out a brief questionnaire regarding screening, the participants were sent a stool collection kit which was then shipped back to the investigators.

Survey Regarding Demographical, Deployment Exposure and Health symptom information

The GWIC participants had to answer to surveys regarding demographics and health condition which included Multi-dimensional Fatigue Inventory (MFI-20), Pittsburg Sleep Quality Index and McGill Pain Inventory \cite{29}, \cite{30}, \cite{31}. The Structured Neurotoxicant Assessment Checklist (SNAC) and Kansas Gulf War and health Questionnaire and Kansas Gulf War Experiences surveys were given to obtain details about self-reported exposures. The survey regarding health condition provided the details if the participants had an ascertained diagnosis of the medical conditions reported by them \cite{26}, \cite{28}, \cite{32}. As part of the call back study, the veterans also filled out questionnaires about their current and recent gut health and use of antibiotics or probiotics.
Collection of Stool Samples from Participants

The GWIC participants of the microbiome study were mailed a Second Genome stool collection kit (Second Genome, San Francisco, CA, USA). The kit was a self-collecting kit which contained a bar coded vial with stabilizing solution for long term preservation of nucleic acids in stool during transportation and storage. Once received from the subjects, the stool samples were stored at -20°C and upon collection of significant sample numbers, they were sent for whole genome shotgun sequencing by COSMOSID. The protocol was approved by Institutional Review Board at Boston University School of Public Health (proposal no. GW170068) on 4/15/2021.

DNA extraction and Whole Genome Shotgun sequencing

Briefly, the total DNA from mouse and human samples were isolated and purified using ZymoBIOMICS Miniprep kit. DNA was quantified using Qubit dsDNA HS assay (Thermofisher, Waltham, MA, USA). Illumina Nextera XT library preparation kit was used with modifications for preparing DNA libraries. Next generation sequencing (NGS) platform was used to perform whole genome shotgun sequencing (WGS), following protocol optimized by vendor.

Metagenomic analysis and assembly

MetaPhlAn v3.0.7 [33] was used to profile the taxonomic composition of each sample with default parameters. The resulting relative abundance tables were then merged with the provided python tool, “merge_metaphlan_tables.py”. A custom python script was used to filter the data to contain only species level identifications and prepare the operational taxonomic unit (OTU) table for statistical analysis. The metaWRAP v1.3.2 [34] pipeline
was used to process and assemble raw sequencing reads from each sample. First, the
“read_qc” module was used with default parameters to trim sequencing adapters and
bases with low PHRED scores. To decontaminate the data, reads mapping to the human
reference genome GRCh38.p12 (RefSeq Acc: GCF_000001405.38) and the mouse
reference genome GRCm38.p6 (RefSeq Acc: GCF_000001635.26) were removed by the
metaWRAP “read_qc” module. Decontaminated reads were used for de novo assembly
using metaSPAdes [35] as contained in the metaWRAP “assembly” module with default
parameters. Resulting contigs were binned by the “binning” module which uses three
binning methods, metaBAT2 v2.12.1 [36], MaxBin2 v2.2.6 [37] and CONCOCT v1.0.0
[38] to produce three sets of bins. The “bin_refinement” module was used to refine these
three bin sets to produce a single set of best bins. Finally, the single bin set was used by
the “bin_reassembly” module which extracts the reads mapping to each bin and uses
them for a second round of de novo assembly to improve the completion and reduce the
contamination of the bins.

Antimicrobial Resistance Gene Family and MGE Identification.

Contigs produced through metaWRAP were used for open reading frame (ORF) finding
using MetaProdigal v2.6.3 [39] with parameters “-c -p meta”. ORFs were then clustered
with CD-HIT v4.8.1 [40], [41] with parameters “-c 0.95 -s 0.90” corresponding to 95%
sequence identity threshold over 90% of the shorter ORF length. Next, ORFs were
mapped to the Comprehensive Antibiotic Resistance Database (CARD) v3.1.0 [42] and
a recently published custom Mobile Genetic Element (MGE) database composed of 278
distinct genes and over 2000 unique gene sequences [15] using the tool “nhmmer” from
the HMMER v3.3.1 [43] software with parameters “-E 0.001 --incE 0.001” corresponding
to an e-value threshold of 0.001 for matches. A custom python script was used to filter multiple hits to select the single best hit for each ORF. Finally, Microsoft Excel was used to generate count data for antimicrobial resistance gene families (AGFs) and MGEs.

Laboratory Methods

Immunohistochemistry

The fixed mouse small intestine and frontal cortex tissues were paraffin embedded and 5 µm thick sections were done for immunohistochemistry. Deparaffinization were performed following previous protocol [44]. Antigen retrieval was performed using epitope retrieval solution and steamer (IHC world, Woodstock, MD, USA). Three percent hydrogen peroxide was used for blocking endogenous peroxidase activity for 20 mins. Serum blocking was performed using 10% goat serum for 1h. Tissue sections were incubated with primary antibodies for IL-1β and BDNF at 1:200 dilution for overnight in humified chamber at 4°C. After incubation, the tissue sections were washed 3 times with PBS containing 0.05% Tween 20 solution. Tissues were probed with species specific biotinylated antibodies (1:200 dilution) followed by incubation with horse radish conjugated streptavidin (1:500 dilution). 3,3’-diaminobenzidine was used as chromogenic substrate solution and counterstaining was performed using Mayer’s hematoxylin. The stained tissues sections were mounted using Aqua Mount (Lerner Laboratories, Kalamazoo, MI, USA). The images were acquired using Olympus BX63 microscope (Olympus, Center Valley, PA, USA). Morphometry were performed using Cellsens Software from Olympus America (Centre Valley, PA, USA).

Quantitative RT-PCR
Quantitative RT-PCR (qRT-PCR) was performed to measure ARG expression in DNA extracted from mouse and human stool samples. Gene specific primers were designed using Primer3 (version 0.4.0) and IDT, purchased from Sigma (St. Louis, MO, USA) (Table S1). SYBR Green Supermix (Biorad, Hercules, CA, USA) was used in CFX96 thermal cycler (Biorad, Hercules, CA, USA). The samples (both mouse and human) were run in triplicates for each gene. Ct or threshold cycle values of all ARG genes were normalized with 16S as internal control. $2^{-\Delta \Delta ct}$ method was used to calculate the relative fold change of the ARGs.

ELISA

Serum IL-6 level was estimated using serum collected from the mice groups using commercially available ELISA kit from Proteintech (Rosemont, IL, USA). The ELISA was performed according to the manufacturer’s protocol.

Statistical analysis

Analyses were performed using R v3.6.3 (1). AGF, MGE, and OTU count data were normalized based on library size using the “estimateSizeFactors” function from the “DESeq2” package [45] with the parameters “type = poscounts”. Normalized count data were then log transformed with the base R function “log2”. PERMANOVA was calculated using the “adonis” function from the package “vegan” (2) with Bray-Curtis dissimilarity and 9999 permutations. Welch two-sample t-test was implemented using the base R function “t.test” with the parameters “conf.level = 0.95, alternative = two.sided” indicating 95% confidence level and two-tailed testing. PCA ordinations of AGF and Taxonomy abundance data were performed using the “rda” function from “vegan”. Procrustes
analysis was performed using PCA ordinations with the function “protest” from “vegan” with 9999 permutations. Distance-based redundancy analysis was performed with the “capscale” function from the “vegan” package with Bray-Curtis dissimilarity. Unpaired $t$-tests (two-tailed tests with equal variance) were performed followed by Bonferroni-Dunn post-hoc analysis to compare between the mouse experimental groups and veteran groups respectively. Chao1 $\alpha$-diversity was calculated using the “chao1” function from the “fossil” package. Correlation analyses between $\alpha$-diversity and selected biomarkers were performed using Pearson’s correlation implemented by the base R function “cor”. All visualizations were rendered using the “ggplot2” package unless otherwise described. For all analyses, $p \leq 0.05$ was considered statistically significant and data are represented as mean ± standard error of mean.

**Results**

Characterization of Anti-microbial resistance in mouse fecal samples

We performed whole genome shotgun sequencing, metagenomic assembly, and functional gene annotation on fecal samples collected from 3 groups: Control, GWI, and GWI_FMT) to construct AGF profiles associated with the GWI mouse model and the fecal microbiota transplant FMT treatment. We detected 95 unique AGFs with an even distribution across all groups with 185 ± 4 (mean ± standard error) total AGFs in Control, 182 ± 3 total AGFs in GWI, and 188 ± 4 total AGFs in GWI_FMT (Fig. 1a). We used permutational multivariate analysis of variance (PERMANOVA) to compare the AGF profiles between sample groups which revealed a significant deviation between the GWI_FMT and GWI ($R^2 = 42.2\%$, $p = 0.002$) and GWI_FMT and Control groups ($R^2 = 41.3\%$, $p = 0.0001$). This analysis indicates that the sample group variable explains
roughly 40% of the variation in the resistome profile. To get a clearer picture of the specific changes in the resistome, we performed differential abundance analysis of the AGFs by manually selecting the AGFs with the greatest variance in relative abundance between sample groups and compared their sum relative abundance as a subset of the resistome (Fig. 1b). These selected AGF groups were significantly increased in the GWI group when compared to the Control group (Welch two-sample t-test, $p = 0.004134$) and between the GWI and GWI_FMT groups (Welch two-sample t-test, $p = 0.00008972$). To explain these changes, we performed procrustes rotation of the principal component analyses (PCAs) of the resistome and microbiome profiles which showed a significant correlation between resistome changes and microbiome composition changes (PROTEST, $M^2 = 0.2834$, $p = 0.0001$, Fig. 1c). Further, distance-based redundancy analysis showed clear segregation of the AGF profiles of each group with that of the GWI_FMT group lying between the Control and GWI groups in the CAP2 axis suggesting that FMT treatment may act to return the resistome to a more Control-like state (Fig. 1D).

To assess the transferability of the AGFs in the microbiome, we also examined the profile of the MGEs constituting the mobilome in each sample group. We detected 67 unique MGEs evenly distributed across all groups with 74 ± 5 total MGEs in Control, 71 ± 3 total MGEs in GWI, and 80 ± 3 total MGEs in GWI_FMT (Fig. 1e). PERMANOVA showed that significant differences in the mobilomes of the GWI_FMT and GWI groups ($R^2 = 42.9\%$, $p = 0.0022$) and the GWI_FMT and Control groups ($R^2 = 30.6\%$, $p = 0.0024$). The relative abundance of manually selected MGEs were also significantly different between Control and GWI groups (Welch two-sample t-test, $p = 0.002101$) and between the GWI and GWI_FMT groups (Welch two-sample t-test, $p = 0.0003543$).
In our analysis of the gut resistome, we detected multiple genes AFGs imparting resistance to antimicrobial classes which have been, marked as highly important and critically important by the World Health Organization WHO (AGISAR, 2018). Overall, glycopeptidase resistance genes were the most common type of AGF followed by quinolones, polymyxins and carbapenem resistance genes. The relative abundance of vancomycin resistance genes were highest (4.44% van-R, 3.57% van-S, 2.33% van-Y and 1.9% van-T) in GWI group compared to Control and GWI_FMT groups (Fig 2a). Interestingly, there were 38 unique genes which were only present in the GWI group but not in the Control or GWI_FMT groups. Of these genes, mcr-1 and ndm-1 drew the most concern because of their high transferability. The Control group also showed maximum resistance towards vancomycin followed by RND (resistance nodulation cell division) efflux pump, MATE transporter and β-lactamase resistance genes. Apart from vancomycin-methicillin resistance, resistance against penicillin, cephalosporins, and macrolides were also observed in GWI_FMT samples. Though these mice were not exposed to any of these antibiotics, and the very fact that they showed resistance towards members of critically important the highest priority group of antimicrobials (AGISAR, 2018, WHO) is a matter of concern.

When comparing the different drug class resistance across the three groups, the majority of the resistome was composed of genes imparting mixed resistance against drug classes with the largest single class being glycopeptide resistance. Genes against this drug class consisted of 4.6% and, 4.4%, and 3.83% of the GWI, Control, and GWI_FMT resistome respectively. Apart from the observed glycopeptide resistance, other similar fractions of
the resistomes detected were in relation to macrolide antibiotics with 4.14%, 4.08%, and 3.74% respectively, peptide antibiotics with 4.06%, 3.94%, and 3.45% respectively, tetracycline antimicrobials 4.08%, 4.01%, and 3.75% respectively and nitroimidazole antibiotics which were present 3.76%, 3.63%, and 3.12% respectively in the GWI, control and GWI_FMT groups (Fig 2b). Multivariate analysis of the profiles of drug class resistances showed significant deviation and correlation dependent on sample groups when comparing the GWI_FMT and Control groups (PERMANOVA, $R^2 = 40.9\%, p = 0.0027$) and the GWI_FMT and GWI groups (PERMANOVA, $R^2 = 56.4\%, p = 0.0025$). Typical gene signatures are identifiable for classes of resistance mechanisms. Resistance mechanism genes such as those which are known to be associated with antibiotic efflux were identified and and made up the highest percentage of all resistomes (20.33%, 20.15%, 19.75% in GWI, control and GWI_FMT respectively) (Fig 2c). Other mechanisms of resistance included target alteration, target protection, and antibiotic inactivation as well as target replacement. When calculating the percentages of these mechanisms, GWI group had the highest value of abundance when compared to the other groups namely, control and GWI+FMT.

We then investigated the presence of different MGEs and MGE types among all of the samples. As ARGs have high propensity to transfer from one bacteria to another, the evaluation of different MGE elements is of utmost importance. Tnp A was most the predominant mobile genetic element present in GWI group followed by IS 91, int2, ISCrspI and intI1 (Fig2d). When comparing different types of MGEs, transposases was found to be the highest in terms of abundance and counts(14.5% in GWI, 14.2% in control and 11.8% in GWI_FMT). Tn 916 (9.3% in GWI), integrase (11.2% in GWI), ISCR (9.57%)
were also present in all the 3 groups of mice (Fig 2e). Multivariate analysis of the MGE type profiles showed a significant deviation and correlation pattern that was dependent on sample groups when comparing to the GWI_FMT and Control groups (PERMANOVA, R^2 = 42.2%, p = 0.0024) and the GWI_FMT and GWI groups (PERMANOVA, R^2 = 52.2%, p = 0.0018).

Characterization of anti-microbial resistance gene in human samples

In order to study whether the results observed on our mouse model were mirrored in the human subjects, we obtained 15 stool samples from GWI veterans (5 in the Hum_Control group and 10 the in Hum_GWI group). We then performed whole genome shotgun sequencing, metagenomic assembly, and functional gene annotation. We detected 108 unique AGFs with 175 ± 3 and 182 ± 3 total occurrences in the Control group and GWI groups respectively (Fig 3a). On comparing the relative abundance of AGFs, we observed a modest but insignificant deviation in the resistome profiles between the two groups (PERMANOVA, R^2 = 7.2%, p = 0.4428). When looking at a more granular scale, there was an insignificant decrease in the relative abundance of selected AGFs (Welch two-sample t-test, p = 0.6874) (Fig 3b). Procustes analysis showed a significant correlation of bacterial taxa and the AGFs (PROTEST, M^2 = 0.5972, p = 0.004) (Fig 3c). Despite minor divergence indicated by multivariate analysis, distance-based redundancy analysis showed clear clustering of the control and GWI groups with minimal overlap on the first two constrained principal coordinates (Fig 3d).

Looking at transferability, we identified 92 unique MGEs across both groups with 77 ± 5 and 85 ± 3 total MGEs in the Control group and the GWI groups respectively (Fig 3e). While there was some deviation in the MGE profiles of the two groups, multivariate
analysis revealed that these changes were not significantly dependent on the sample groups (PERMANOVA, R^2 = 6.5%, p = 0.599). Interestingly, the relative abundance of selected MGEs were higher in GWI group compared to the Control, however, this difference was not statistically significant (Welch two-sample t-test, p = 0.05331) (Fig 3f).

Distribution of selected ARGs and MGEs in human samples

To check the distribution of ARGs and MGEs in human samples, identical analysis was carried out in the 2 groups of human data. Ciprofloxacin antibiotic efflux pump showed the highest relative abundance in GWI (5.26%) followed by RND antibiotic efflux pump (4.16% in GWI), vanR (3.96% in GWI) and tetR (3.65% in GWI) (Fig 4a).

Comparing the drug class resistances of the 2 groups, glycopeptide antibiotic was most predominant followed by macrolide, tetracycline, fluroquinolone and glycycline antibiotics. The percentage of the resistance were observed in the sequence of 4.08%, 4.01%, 3.91% and 3.1% respectively. The profiles of drug class resistances did not significantly deviate or depend on the sample group (PERMANOVA, R^2 = 6.4%, p = 0.6181) (Fig 4b)

A total 9 different types of resistance mechanisms were detected, in which antibiotic efflux pump (20.72 % in GWI and 20.76% in control) was the most common. The mechanisms of antibiotic target alteration, protection, inactivation and replacement mechanisms were also detected across the 2 groups (Fig 4c).

Among the 31 unique different MGE groups studied, tnp A was found to be most predominant (7.8% in GWI, 8.8% in Control). A total of 23 MGEs were found to be present in the GWI group. Int2, IS 91, IS 621 and MGE 10 were found to be present in both groups (Fig 4d). We also observed 8 different MGE types unique in GWI group but
transposases (13.63% in GWI and 12.71% in control) and integrases (10.67% in GWI and 9.97% in control) were found to be the most abundant in all MGE types (Fig 4e). Multivariate analysis of the MGE class profiles revealed moderate deviation between sample groups which was shown to be independent when compared between sample groups (PERMANOVA, R^2 = 12.1%, p = 0.0632).

Expression study of antimicrobial resistance genes in mouse and GWI veteran samples by q-RTPCR analysis

Based on the host microbial genome analysis in the mouse model we selected 13 different resistant genes when compared between mouse GWI and mouse Control (AGF count cut off value-1), 19 different resistant genes when compared between mouse GWI and mouse GWI_FMT (AGF count cut off value 1) and 10 when compared between GWI-veteran and control-veterans (AGF count cut off value-3).

The expression of VANU gene was 6 fold higher in GWI mouse group when compared to the mouse control group. The rifampin phosphotransferase (RP) gene also showed a 6 fold increase in mouse GWI group followed by AAC3' (5.56 fold), NDM (4.45 fold), APH6 (3.6 fold), MCRK (2.66 fold), MCRE (2.3 fold) when compared to subsequent controls. The estimation of the expression of resistant genes against mouse GWI and GWI_FMT groups revealed that AAC3' expression was highest with fold change of 7.23 when compared to the FMT group. OXA β-lactamase (OXA) also showed fold change of 4.5, while FONA β-lactamase (FONA) showed a change of 3.15 fold when compared to the FMT group. Subsequently, the expression of BAH amidohydrolase (BAH) and class C LRA β-lactamase (LRA) were increased by 3.1 and 2.15 fold when compared to FMT
groups suggesting that fecal microbiota transfer was efficient in decreasing the
abundance and expression of these antimicrobial resistance genes (Fig 5a & b).

Comparison of microbiome sequencing data in human samples showed that there was
a huge increase in expression of isolucyl t RNA synthetase (ILES) (267 fold), phosho-
ethanolamine transferase (PMR) (162 fold), RND efflux pump (RND) (82 fold) followed by
rifamycin resistant (RPOB) and chloramphenicol acetyl-transferase (CAT) (37 fold) in the
GWI group when compared to controls suggesting that the present day Gulf war veteran
with CMI had a significant alteration of antibiotic resistance pattern when compared to
controls (Fig 5c).

Gastrointestinal, systemic and neuronal inflammation and its association with ARGs and
Drug Classes in mouse GW-CMI samples

Gastrointestinal and neuronal inflammation is reported in deployed GW veterans with CMI
also termed as GWI and in preclinical GW-CMI mouse models due to the influence of GW
chemicals ([26], [46], [47], [48], [49], [50]. Increase in systemic inflammatory markers were
also reported in GWI veterans and mouse models [51], [26], [50], [52]. Following our study
of the expression of AGFs and MGEs, we focused our study to examine whether an
association exists between AGFs and MGEs and biomarkers of GWI pathology thus
suggesting a role of gut resistome in influencing the host health. The purpose was also
to show whether a predictive insight can be made to future susceptibility to infectious
disease related to hospital acquired infections in the veterans, elderly and
immunocompromised individuals. Results in mice showed that the expression of IL-1β in
small intestine significantly increased in GWI group (p<0.000001) compared to the
Control group, as shown by immunoreactivity of the cytokine in the villi (Fig 6a,b).
Treatment with FMT significantly decreased (p<0.000001) the expression of IL-1\(\beta\) in GWI_FMT mice groups compared to GW group (Fig 6a,B). To study the association between IL-1\(\beta\) expression and AGF diversity, we performed a correlation analysis. Results showed a positive correlation (r= 0.8463, p=0.034 & r=0.9691, p=0.001 respectively) between \(\alpha\)-diversity of AGFs, resistant drug classes and IL-1\(\beta\), suggesting that alteration of gut resistome had a significant correlation in gastrointestinal inflammation (Fig 6f).

We observed a significant increase (p=0.000413) in serum IL-6 level in GWI mouse group when compared to Control and GWI_FMT group (Fig 6c). Results also suggested that increased \(\alpha\)-diversity of AGFs and resistant drug classes positively correlated (r=0.8043, p=0.196 & r=0.9669, p=0.033) with increased systemic IL-6 level in GW mouse group (Fig 6g).

Our previous studies have reported that a decrease in synaptic plasticity marker BDNF played a key role in brain pathology in GW chemical exposed mice [44] Results showed that expression of BDNF significantly decreased (p=<0.00001 between Control vs GWI & GWI vs GWI_FMT) in GWI group when compared to Control and GW_FMT mice groups (Fig 6d). Interestingly, a negative correlation (r=-0.8866, p=0.19 & r=-0.9799, p<0.001) was observed between BDNF and AGFs suggesting that increased AGF-\(\alpha\)-diversity may have a strong influence on observed neuroinflammation GWI mice group (Fig 6j)

Discussion
We report a novel alteration of host gut resistome in chronic multisymptom illness as observed in a subsection of Gulf War veterans. We also linked the association of systemic inflammation and an altered gut resistome that is linked to increased circulating proinflammatory cytokine IL6, intestinal pathology and neurotrophic factor BDNF. IL6 has been shown to be a pleiotropic cytokine and is key to gastrointestinal disturbances, and cognitive deficits [53], [54]. Recent advances in understanding the host resistome improved our knowledge about evolution, origins and emergence of antibiotic resistance though the field is still evolving [55]. Previously, our knowledge of resistome included proto-resistance and silent resistance genes [55]. Silent-resistance genes do not cause phenotypic resistance until they are transferred via MGEs or a mutation occurs in the associated regulatory elements. Gut microbiome is usually associated with a silent resistome, as they have the potential to contribute in clinical resistance through mobilization [55]. Changes in the host environment has a direct effect on human microbiome that leads to the enrichment of ARGs [12, 56]. Association to MGEs increases the susceptibility to antibiotic resistance of the host gut microbiome. Sun et al., reported that the gut microbiome and resistome of veterinary students visiting a swine farm had been significantly altered after their 3 months visit which was partially restored even after 4-6 months post visit [12]. A recent report suggested that COVID-19-associated travel norms had greater impact on gut microbiota and ARGs than did pre-pandemic international travel [57]. As a result, the phylum *Actinobacteria*, known to be a resident of the gut, decreases while the resistance against β-lactams, polystyrene increases thus impacting the gut physiology [57]. Our study supports the existing evidence that the resistome is highly dependent on the gut microbiome and proves a strong association
between changes in the bacterial taxa and the gut resistome, particularly in GWI groups, both in the case of mouse models and veterans with GWI as studied by PERMANOVA and procustes analysis. Interestingly, we found that the richness of ARGs increases with the GW chemical exposure early in the life cycle.

Previous reports stated that elderly individuals and veterans had increased resistance to sulfonamide, macrolide, β-lactam antibiotics followed by tetracycline as well as fluoroquinolones especially found in Acinetobacter baumannii isolates [58], [59]. Reports also stated that fluoroquinolones and cephalosporin usage should be prescribed in a limited manner among the elderly and veterans as they have higher susceptibility in developing resistances upon treatment with such high generation antibiotics [59]. However, there is very little evidence about the ARGs in veterans, especially GW veterans suffering from CMI or collectively referred as GWI who were deployed 30 years back and are presently in the age range of 50-60 years. The present study is the first to report the alteration of ARG signature in GW-CMI veterans as well as in preclinical CMI mouse model closely related to GW which mimics the present health condition of the GW-CMI veterans. We identified that glycopeptide resistance is high in GWI compared to other mouse groups following macrolide, peptide and tetracycline antibiotics. This study also suggests that GW chemical exposure may be responsible for the alteration and appearance of ARGs and MGEs in mice since they have been maintained in a controlled environment with very few risk of exposure to a multifaceted environment. A further study of the resistant drug classes as well as mechanisms of resistance showed that antibiotic efflux is the major mechanism of resistance in both mouse and GW-CMI veterans. The
above mechanism further aids the transfer of the glycopeptide antibiotic efflux and may act as a signature resistance mechanisms associated with GW-CMI pathology.

FMT has been reported to restore the beneficial microbiome in pathological conditions like irritable bowel syndrome, type 2 diabetes, nonalcoholic steatohepatitis, neurological disorders and type 2 diabetes where gut microbial dysbiosis plays a direct role in the disease progression [60], [61]. Studies have also reported that FMT administration have decreased the ARGs in patients with cirrhosis and Clostridium difficile infection[61]. In the present study, we treated GW chemical exposed mice with FMT in GWI_FMT group. We observed a significant decrease in relative abundance of selected AGFs and MGEs in GWI_FMT. Moreover, the results showed an increase in unique AGFs and MGEs in GWI_FMT which may be transferred from the resident commensal microbiome of the donor mice group. This also emphasizes the need for screening the fecal samples of healthy donors for FMT [62].

One of the important highlights of this study is the accumulation of ARGs in GW-chronic multisymptom illness group that have high transferable capacity. This transfer may occur through horizontal gene transfer (HGT) method based on MGE patterns. MCR phosphoethanolamine transferase and NDM beta-lactamase are two such the genes that are present in GWI mouse group but not in Control and GWI_FMT group. As mentioned previously, these mice were never exposed to any kind of antibiotics (β-lactams or colistin) during the entire course of the study, but they showed a spontaneous acquisition of AGFs in GW chemical exposed group. This may be due to intrinsic transfer or extrinsic transfer through HGT. We were able to confirm the presence of these genes by sequencing (data not shown), but further studies are needed to study their mechanism of
transfer. Another limitation in our observation of gene transfer may be from the use of diet in these mice. The mice in our study were fed with the standard chow diet though in a consistent manner in all groups. Diet induced acquisition of microbial resistance genes should be studied as a mechanism of acquiring antibiotic resistance genes in all further studies though the chemical exposure was the principal variable that differentiated the groups.

Here in this study, we were able to identify diverse types of MGEs in which tnpA is most abundant followed by transposase. Another study by Parnanen et al. stated that transposase constitute most abundant MGE class in all samples [15]. This study completely is consistent with our observations about MGEs. Higher abundance of MGEs belonging to transposon group indicated that there is a higher potential for ARG transfer in GW-CMI pathology. To track the ARG transfer through MGE, detailed investigation is needed whether these genes can transfer through any transferrable plasmids or by any other IS element/ transposons.

Studies have also reported that low dose antibiotics have increased the abundance of a single pathogenic bacteria, however, difference in ARGs were not significant compared to control groups [63]. This study also showed change in immunological markers but association between ARGs and immunological markers have not been established [63]. Also, IBD phenotype is known to be associated with microbiome dysbiosis which led to upregulation of antibiotic resistance [64], a condition also observed in GW-CMI veterans [65]. We have shown a direct association between gastrointestinal, systemic and neuronal inflammation observed in GW-CMI pathology and diversity of AGFs and resistant drug classes in a mouse model. Interestingly, association between diversity of
AGFs and systemic proinflammatory marker IL-6 will be an important benchmark in future studies of chronic multisymptom illness and other related pathology. Further, mechanistic studies are needed to establish the exact role of ARGs in GWI pathophysiology and can be aided by GW-CMI veterans studies where probiotics or microbiome restoring short chain fatty acids (butyrate) is used as a therapy.

In conclusion, our study revealed for the first time that exposure to GW chemicals associated with CMI caused significant changes in ARGs and MGEs in GW-CMI mouse model and veterans alike. Further, a strong association was established between an altered resistome and systemic IL6 levels in a translatable mouse model that has broad implications in the general population suffering from similar ailments. Strikingly it is expected that a strong 78 million of the US population will be elderly category by 2030. Most of them have a history of prolonged antibiotic use, a case similar with our aging veterans. The scenario is also striking owing to old age associated hospitalizations and increased chances of hospital acquired infections. In addition, FMT can be used as a therapeutic strategy against the increased antibiotic resistance in veterans and elderly to attenuate a possible altered resistome. A focused study on each of the antibiotic resistant genes that was altered in the CMI mouse model and its modulation by FMT or in GW-CMI veterans can be an avenue for drug discovery and personalized medicine in future.

Conflict of Interest

The authors declare no conflict of interest.
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Author contributions:
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Data availability and resources: Microbiome and resistome sequence data that support the findings of this study have been deposited in GenBank with the accession code: PRJNA734321 with the link: https://www.ncbi.nlm.nih.gov/sra/PRJNA734321

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Figure Legends:

Fig. 1 Distribution of ARGs and MGEs in mouse models. a. Boxplots showing total abundance of antimicrobial gene families (AGFs) in Control (wild type mice administered with vehicle, n=6), GWI (GW chemical exposed persistence mice group, n=6) and GWI_FMT (persistent mice group exposed to GW chemicals followed by FMT treatment, n=6). Boxes indicate interquartile range (n=6 biologically independent in each group). Median values of 187 in Control, 185 in GWI, and 190 in GWI_FMT indicated by solid black lines. Error bars extend to the most extreme values within 1.5 time the interquartile range. Differences between mean total AGFs detected shown to be insignificant (Control – GWI p = 0.557, GWI – GWI_FMT p = 0.312) by Welch two sample t-test. b. Comparison of the relative abundance (%) of selected AGFs between (i) GWI and Control groups and (ii) GWI and GWI_FMT groups. c. Procrustes rotation analysis comparing resistome (blue) and microbiome (red) changes using PCA ordinations, (X axis: PCA1, PCA2-Y axis,
PROTEST, $M^2 = 0.2834$, $P= 0.0001$). d. dbRDA of Bray-Curtis dissimilarity between ARG and Taxa across sample groups. Constrained principal co-ordinates CAP1 (35.6 %) and CAP2 (4.2 %) are calculated to explain 39.8 % of variance detected. L ines connect points from the center of gravity of each sample group. e. Boxplot of total abundance of MGEs with median values 72 in Control, 72 in GWI, and 80 in GWI_FMT. f. Relative abundance (%) of selected mobile genetic elements (MGEs) i. Control and GWI, ii. GWI and GWI_FMT

Fig. 2 Classification of selected AGFs and MGEs. a. Group bar chart showing relative abundance (%) of selected AGFs in Control, GWI, and GWI_FMT. Stacked bar analysis of relative abundance (%) of b. drug classes resistances and c. mechanisms of resistance. Relative abundance (%) of d. MGEs and e. MGE types.

Fig. 3 Distribution of AGFs and MGEs in GWI-veteran groups. a. Change in total abundance of AGFs in Hum_Control (veteran group without GWI symptom, n=5) and Hum_GWI (veteran group with GWI symptom, n=10) with median values of 177 in Hum_Control and 181 in Hum_GWI. Differences in total abundance values were shown to be insignificant ($p = 0.1001$) by Welch two-sample $t$-test. b. Comparison of relative abundance (%) of selected AGFs between Hum_Control and Hum_GWI groups. c. Procrustes rotation analysis comparing resistome (blue) and microbiome (red) changes using PCA ordinations, (X axis: PCA1, PCA2-Y axis, PROTEST, $M^2 = 0.5972$, $P= 0.0028$). d. dbRDA of Bray-Curtis dissimilarity between AGFs and Taxa. Constrained principal co-ordinates CAP1-(6.5 %) and CAP2 (18.6 %) account for variance 25.1 % of detected variance. e. Total abundance of MGE element with median values 80 in Hum_Control and 85 in Hum_GWI. f. Relative abundance (%) of selected MGEs in Hum_Control and Hum_GWI

Fig. 4 Classification of selected AGFs and MGEs in veterans. a. Grouped bar chart showing relative abundance (%) of AGFs in Hum_Control and Hum_GWI. b. Stacked bar analysis of
relative abundance (%) of b. drug classes and c. mechanisms of resistance. Relative abundance
(%) of d. MGEs and e. MGE types.

Fig. 5 Relative expression levels of antimicrobial resistance genes: mRNA expression of
AGFs a. Between Control and GWI b. Between GWI and GWI_FMT c. Between Hum_Control
and Hum_GWI groups. The mRNA expression was calculated as fold change against control (in
5a), against GWI_FMT (in 5b) and Hum_Control (in 5c). All data are represented as mean ± SEM.
Statistical significance was analysed using unpaired T-test where *p<0.05, **p<0.01, ***p<0.001.

Fig. 6 Gastrointestinal, systemic, brain inflammation and its correlation with antibiotic
resistance genes and the drug classes in GWI mouse model. (a) Representative
immunohistochemistry image showing immunoreactivity of proinflammatory cytokine IL-1β
marked by red circle) in Control (wild type mice administered with vehicle, n=6), GWI (GW
chemical exposed persistence mice group, n=6) and GWI_FMT(persistent mice group exposed
to GW chemicals followed by FMT treatment, n=6). Images were taken at 20X magnification. (b)
Bar graph depicting immunoreactivity of IL-1β. Results are represented as mean±SD of %ROI
(mean value calculated from 2 different fields in each sample). Statistical significance was
analyzed by unpaired t-test where *p<0.05, **p<0.01, ***p<0.001. (c) Bar graph depicting the
serum IL-6 level at pg/ml in Control, GWI, GWI_FMT mice groups. Statistical significance was
analyzed by unpaired t-test where *p<0.05, **p<0.01, ***p<0.001. (d) Representative
immunohistochemistry image showing immunoreactivity of synaptic plasticity marker BDNF
marked by red arrows) in Control, GWI, GWI_FMT mice groups. Images were taken at 20X
magnification. FC - Frontal Cortex, HC – Hippocampus. (e) Bar graph depicting immunoreactivity
of BDNF. Results are represented as mean±SD of %ROI (mean value calculated from 2 different
fields in each sample). Statistical significance was analyzed by unpaired t-test where *p<0.05,
**p<0.01, ***p<0.001. (f) Correlation plot between α-diversity (Chao1) of resistant AGFs and drug
classes and immunoreactivity of IL-1β in mouse GW group in small intestine section. Pearson’s
linear regression is shown in red with 95% confidence bands. (g) Correlation plot between α-
diversity (Chao1) of resistant AGFs and drug classes and serum IL-6 level in mouse GW group.

Pearson’s linear regression is shown in red with 95% confidence bands. (h) Correlation plot
between α-diversity (Chao1) of resistant AGFs and drug classes and immunoreactivity of BDNF
in mouse GW group in brain section. Pearson’s linear regression is shown in red with 95%
confidence bands.
Fig. 2

Sample Group: Control, GWI, GWI_FMT

- Glycopeptide resistance gene cluster (vanR)
- Glycopeptide resistance gene cluster (vanS)
- Major facilitator superfamily (MFS) antibiotic efflux pump
- Resistance-nodulation-cell division (RND) antibiotic efflux pump
- Glycopeptide resistance gene cluster (vanW)
- General Bacterial Porin with reduced permeability to beta-lactams
- Glycopeptide resistance gene cluster (vanXY)
- General Bacterial Porin with reduced permeability to beta-lactams; resistance-nodulation-cell division (RND) antibiotic efflux pump
- Lipid A phosphatase
- Methicillin resistant PBP2
- APH(3')
- Glycopeptide resistance gene cluster (vanX)
- Glycopeptide resistance gene cluster (vanU)
- Bacterial amidohydrolase
- Rifampin ADP-ribosyltransferase (Arr)
- BJP beta-lactamase

Relative Abundance (%)

b) Drug Class

- Mixed
- Acridine dye
- Aminoglycoside antibiotic
- Antibacterial free fatty acids
- Carbapenem
- Cephalosporin
- Cephamycin
- Fluoroquinolone antibiotic
- Glycopeptide antibiotic
- Macrolide antibiotic
- Nitrimidazole antibiotic
- Pem phenolic peptidic antibiotic
- Tetracycline antibiotic
- Triclosan
- Nucleoside antibiotic
- Rifampicin antibiotic

Relative Abundance (%)

Drug Class: Control, GWI, GWI_FMT

Resistance Mechanisms

- Antibiotic efflux
- Antibiotic efflux; antibiotic target alteration
- Antibiotic efflux; reduced permeability to antibiotic
- Antibiotic target inactivation
- Antibiotic target alteration
- Antibiotic efflux; reduced permeability to antibiotic
- Antibiotic target protection
- Reduced permeability to antibiotic

Relative Abundance (%)

MGE

- Int-Tn916
- int3
- IS621
- IS610
- ISCR8
- ISCrsp1
- ISPtrmu1
- ISPtrv1
- istA
- ISUnb4
- orf513
- qacEdelta
- rep19
- rep24
- Tn916-orf18
- Tn916-orf5
- Tn916-orf6
- Tn916-orf7
- Tn916-orf8
- Tn916-orf9
- TnpA
- TnpA1
- TnpA4
- TnpAc
- Xis-Tn916

Relative Abundance (%)

MGE Type

- insertion_element_IS911
- integrase
- IS262
- IS610
- ISCR
- ISPmu1
- ISPv1
- istA
- istB
- ISUnb4
- orf513
- plasmid
- qacEdelta
- Tn916
- transposase
Fig. 5

(a) Normalized mRNA expression (x fold change control) in GWI vs. CONTROL.

(b) Normalized mRNA expression (x fold change gw1.fmt) in GWI vs. GWI_FMT.

(c) Normalized mRNA expression (x fold change human control) in HUM_GWI vs. HUM_CONTROL.
Fig. 6
### List of primers for Real time PCR analysis:

**a. CONTROL and GWI:**

| SL no. | Gene name and description | Abbreviation | Forward | Reverse | Ta  |
|--------|---------------------------|--------------|---------|---------|-----|
| 1      | General Bacterial Porin with reduced permeability to beta-lactams (GBP) | GBP         | TAACGATCAGCTTTGGTCTG | CTTGAAAGCACCCTTTGTCA | 56.3 |
| 2      | APH(3') R/P               | APH(3')     | GCATGAGGCTCTTCACTCC | GGCGCAGATCTTTATTCAGT | 55.7 |
| 3      | MCR phosophoethanolamine transferase MCR R/P | MCRK/E     | AAATTGCGAGTACGACACC | GCTGCTCCTGGGTGTTTTTA | 56.3 |
| 4      | rifampin phosphotransferase RP R/P | RP         | TTCCGACTTGAGAGACACCT | GTCCGTTAGATCGCCCAATGTT | 56.0 |
| 5      | Glycopeptide resistance gene cluster;vanU | vanU       | TCAGGAGGTCTGGAGTAA | TCGCAAAACTCAAATTTCAA | 55.8 |
| 6      | Methicillin resistant PBP2 | PBP2       | GTCCAATCGGTTGGTTTG | TCTGGCAAAACTTTCTCGAT | 56.2 |
| 7      | APH(6)                    | APH(6)      | ATCGCTTTGCTGCTTTTA | ATGTGACAGATCAGCCATTA | 56.2 |
| 8      | BjP beta-lactamase        | BjP         | TCAAGCTCATCTCAACACG | TTTTCTGCAACCGGATAG | 55.9 |
| 9      | Lipid A phosphatase       | LAP         | GCCGTACCTACTCGCATCA | GCCGTAGACGACCTTTCACT | 56.1 |
| 10     | AAC(3)                    | AAC(3)      | ATAGCCGCGAGTCTGTGTA | GAGGATCGGTGTGCTTAGG | 55.5 |
| 11     | NDM beta-lactamase        | NDM         | ATATCACCCTGGAGTACGAC | TATGATCGATCGGCATCA | 56.0 |
| 12     | Lincosamide nucleotidyltransferase (LNU) | LNU | AGCGTTCAAACCAAGCAATG | CTGCAACGACACTTTTCGTA | 55.9 |
| 13     | Glycopeptide resistance gene cluster;vanX | vanX       | TGCTCTTATGGGACGGCTAC | AAGCCACATACCCCTTTCG | 56.5 |
| 14     | Erm 23S ribosomal RNA methyltransferase (ERM) | ERM       | ATATGATCGACGAGTACCTT | ATCCACTGCGAGACTCTT | 56.1 |

**b. GWI and GWI_FMT:**

| SL no. | Gene name and description | Abbreviation | Forward | Reverse | Ta  |
|--------|---------------------------|--------------|---------|---------|-----|
| 1      | Glycopeptide resistance gene cluster;vanRA | VAN RA     | CAGTGGGATAAGGAGCAGAA | TCGGTGGAGTAAGGGATAA | 54.6 |
| 2      | Glycopeptide resistance gene cluster;vanRM | VAN RM     | GTCAAGTCGCTGACAGTGATAA | GTTCAACGAGACAACATTC | 55.3 |
| 3      | Glycopeptide resistance gene cluster;vanS | VANS       | TCACGGTTGG ACCAGGCTAC | GGTCAACTGCAAAGCATATAG | 56.5 |
| 4      | Major facilitator superfamily (MFS) antibiotic efflux pump; resistance-nodulation-cell division (RND) antibiotic efflux pump | MFS/RND  | AACTCGCGCGGTCAATATAA | GGCCAGGTTTCTGATCAA | 56.1 |
| 5      | Glycopeptide resistance gene cluster;vanT | VANT       | ATACCGCGCATACGAGATA | TAAATGTAATTTGATGCCGT | 56.7 |
| 6      | kdpDE                     | KDPDE       | CAGCGTATCAGTCGTTTCTTCT | GGCGGAGATCGAGAAATCAA | 56.7 |
| 7      | Multidrug and toxic compound extrusion (MATE) transporter | MATE       | CGCTGAAATTTGCGCATATTAC | CGACAAACATTTGCGTGATAG | 58.3 |
| 8      | BlaZ beta-lactamase       | BLAZ        | TGCCGCTTTGATCGGAATTAG | CTAAAGCACAACGACTGAT | 56.6 |
| 9      | General Bacterial Porin with reduced permeability to beta-lactam | RND        | GTCACGTGAAAAAGTCTAGAGC | ATACATCCCGACGCCGTAAG | 56.3 |
| SL no. | Gene name                                           | Abbreviation | Forward              | Reverse                   | Ta  |
|-------|----------------------------------------------------|--------------|----------------------|---------------------------|-----|
| 1     | ATP-binding cassette (ABC) antibiotic efflux pump | ABC          | GAAGG GCCAAAGAGCTATTG | CAGAGATGGAAGGGCTAAA       | 55.8|
| 2     | glycopeptide resistance gene cluster;vanR         | VANR         | CACTTCTGCGGGAGGATT    | GTTAAGGTTCCTGCTTTGTCTG    | 55.9|
| 3     | resistance-nodulation-cell division (RND) antibiotic efflux pump | RND          | GATCTTGACTCCAGCCTTTTG | CTCTTCTCAACATGCGTAAA     | 55.8|
| 4     | tetracycline-resistant ribosomal protection protein | TET          | CCTGTCGCGGAGAAATGTAT  | CTCTACCGTGTCGCGAAGAAT     | 56.9|
| 5     | major facilitator superfamily (MFS) antibiotic efflux pump | MFS          | GCAATGCGGAGACTCAAAC   | CCCTATCGCTACGGCAATAAT     | 56.6|
| 6     | pmr phosphoethanolamine transferase                | PMR          | CTCGAGTGTTATCCCGTTAT  | CAGCAGGATCTCATAGCTCTTTCC  | 56.1|
| 7     | glycopeptide resistance gene cluster;vanH         | VANH         | GCTTTGATGCTGATGCTGATG | GTCATATCCCAGCTCTTT        | 56.8|
| 8     | antibiotic-resistant isoleucyl-tRNA synthetase (IleS) | ILESH        | CCTACTACTTCTCCACGCTTATG | CAGCAGGTAACGTCCATCTC      | 54.7|
| 9     | rifamycin-resistant beta-subunit of RNA polymerase (rpoB) | RPOBH        | ACCTTCTCCAGCCTGACTTT  | CACGGTCTGGGACTTTGATT      | 55.1|
| 10    | chloramphenicol acetyltransferase (CAT)           | CATH         | CAGGTAAGAACCGAAACATCA | TGCGATCTGGCAAGAGAAAG      | 56.4|