Environmental Microbiome

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Targeted probe capture metagenomics-enabled surveillance of multidrug-resistant organisms and antimicrobial resistance genes in post-handwashing areas of public washrooms

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Abstract

Background Public washrooms (toilets) are potential hubs for pathogen transmission, yet the risk of microbial re-contamination via post-handwashing surfaces remains understudied. We characterized the prevalence and distribution of multidrug-resistant organisms (MDROs) and antimicrobial resistance genes (ARGs) in post-handwashing areas by sampling four high-contact sites, including faucets, paper dispensers, hand dryers, and exit door handles, in public washrooms across healthcare, commercial, and recreational facilities.

Results From the 232 post-handwashing surface samples collected, we isolated 17 MDROs (7.33% prevalence) from cultures, including extended-spectrum beta-lactamase (ESBL)-producing *Enterobacterales* (ESBL-E, $n=10$), carbapenem-resistant *Pseudomonas aeruginosa* (CRPA, $n=5$), and methicillin-resistant *Staphylococcus aureus* (MRSA, $n=2$). Additionally, we novelly employed targeted probe capture metagenomics (TCM), which utilizes oligonucleotide probes to enrich and detect low-abundance microbial species and ARG sequences. TCM revealed the detection of human pathogenic taxa in 65.2% of samples, including *P. aeruginosa* (78.4%), *Acinetobacter baumannii* (77.9%), and *S. aureus* (71.1%). Clinically critical ARGs, such as bla*CTX-M* (2.0%), bla*NDM* (2.9%), bla*SHV* (3.4%), and *mecA* (62.3%), were detected in 63.7% of samples, indicating a potential transmission within the post-handwashing area.

Conclusions Our findings highlight the role of post-handwashing areas as underrecognized reservoirs for MDROs, particularly MRSA. Furthermore, this study demonstrates the utility of TCM in public health surveillance by enabling a sensitive detection of rare but high-risk microbial species and drug resistance determinants in low-biomass environmental samples. This study offers a comprehensive and nuanced view of the microbial and resistome landscape of washroom environments, offering a revolutionary approach for future environmental surveillance.

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Keywords Pathogen surveillance, Multidrug-resistant organisms, Antimicrobial resistance genes, Targeted probe capture, Metagenomics, Urban microbiome, Urban resistome

Background

The emergence and global dissemination of multidrug-resistant organisms (MDROs) and antimicrobial resistance genes (ARGs) represent a critical public health crisis, complicating the treatment of infectious diseases and causing millions of deaths each year [1]. Public washrooms, characterized by the high number of users and persistently humid environments, are underrecognized reservoirs for drug-resistant microorganisms [2, 3]. While handwashing remains a cornerstone of personal hygiene, post-handwashing areas, where individuals may inadvertently re-contaminate cleansed hands, are much-neglected as potential reservoirs of MDROs and ARGs. Understanding the microbiological and antimicrobial resistance (AMR) landscapes of post-handwashing areas is crucial to evaluating the efficacy of current hygiene practices and developing effective infection control strategies.

Public washrooms pose elevated infection risks compared to private washrooms due to their greater microbial diversity and high foot traffic [4]. Post-handwashing surfaces, including faucets, paper dispensers, hand dryers, and exit door handles, serve as critical junctions for microbial exchange [5]. Studies demonstrate that damp hands transfer microorganisms to surfaces and become re-contaminated more readily than dry hands [6], with high-touch surfaces acting as hubs for pathogen transmission [7]. Notably, drug-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), are frequently isolated from washroom surfaces, potentially causing infections to washroom users [8–10].

Pathogen transmission in washrooms primarily occurs through surface (fomite) contact and aerosol-mediated respiratory routes [5]. Direct contact with contaminated surfaces is a key exposure pathway for pathogen transmission in public washrooms [4]. This occurs through the surface-to-surface spread by hands or bacterial aerosolization followed by surface deposition. High-touch surfaces, such as flushing buttons and door handles, were identified as hotspots for pathogen transmission in public washrooms [2, 11]. Moreover, a plethora of studies have also shown that the washroom surface harbors a diverse array of pathogens associated with fecal–oral transmission. Fecal organisms, such as *Escherichia coli*, *Shigella* spp., and *S. aureus*, can persist on surfaces for days to months [12–14]. Furthermore, the risk of infection via fomite contact is amplified, particularly with low-infection-dose pathogens, such as norovirus, rotavirus, and enterohemorrhagic *E. coli* (EHEC) [15, 16]. Aerosolization represents another key transmission route, driven by toilet flushing, handwashing, hand drying with jet air dryers, sneezing, coughing, and vomiting [11, 16, 17]. These activities generate and disperse pathogen-containing droplets and aerosols into the washroom air, posing risks of respiratory infection to washroom users through inhalation or surface deposition, which can contaminate hands through contact [18]. For instance, respiratory viruses, including SARS-CoV-2 and adenovirus, have been detected in washroom air in hospital and community settings [19, 20]. Additionally, higher levels of MRSA, a frequent cause of hospital-acquired pneumonia, have been recovered from air samples of hospital washrooms equipped with jet air dryers compared to those using paper towels [21]. Collectively, these findings highlight the combined risks of fomite- and aerosol-mediated transmission in washrooms, particularly in areas utilizing hand-drying facilities that disperse pathogen-containing aerosols.

Conventional pathogen surveillance, relying on culture-based methods and quantitative PCR (qPCR), provides valuable insights into pathogen viability and target-specific detection. However, their utility in environmental surveillance is constrained by a narrow analytical scope, such as a predefined set of limited targets, and dependence on a priori knowledge like primer sequences [22]. Unbiased metagenomics, while offering a hypothesis-free approach to analyze all genetic materials in a sample and thus the simultaneous detection of bacterial, viral, and fungal species, however, struggles to detect low-abundance pathogens and ARGs in environmental samples due to the low-biomass environment and overwhelming background DNA from various sources (e.g., human, insects, plants) [23]. Targeted probe capture metagenomics (TCM) addresses these limitations by utilizing oligonucleotide probes to selectively capture thousands of microbial genes and ARGs from complex and low-biomass samples. This procedure enhances the proportion of target genes by several orders of magnitude, thus enhancing the signals of low-abundance reads and improving their detection [24]. This technique enables a high-throughput, high-resolution genomic profiling, making it particularly effective for capturing targets in low-biomass samples (e.g., air, surfaces) [25]. Although the utility of TCM in pathogen and AMR surveillance was demonstrated in complex matrices like foods [26, 27], wastewater [28, 29], and wildlife [30], its applications to urban built environments, such as hospitals, workplaces, and public transport systems, remain unexplored, let alone public washrooms. Built environments present unique challenges and opportunities in public

health surveillance, as their host microbial communities were shaped by diverse factors including anthropogenic activities, building designs, construction materials, and indoor and outdoor conditions [31, 32]. Applying TCM to these settings could uncover hidden AMR reservoirs, inform public health strategies, and ultimately advance our understanding of microbial and AMR dynamics in urban spaces, and contribute to a more effective infection control measure.

To our knowledge, this study provides the first TCM-enabled surveillance of MDROs and ARGs on the post-handwashing surfaces in high-risk public washrooms (e.g., hospitals, catering outlets, wet markets). By integrating culture-dependent and metagenomic approaches, we delivered a comprehensive characterization of the microbial and AMR landscape of washroom environments. This work may contribute to developing evidence-based disinfection protocols, mitigating community-acquired AMR risks, and strengthening pandemic preparedness of existing public health surveillance frameworks.

Methods

Sample collection and processing

Post-handwashing surface samples were collected from public washrooms located in hospitals, catering outlets, transportation systems, school campuses, hotels, wet markets, and recreational facilities in Hong Kong between July and October 2024. A summary of surface samples collected ($n = 232$) was provided in Supplementary Table S1.

Polywipe™ (Medical Wire & Equipment, UK), a pre-moistened sponge swab, was used for the microbial surface sampling at all sites. At each public washroom, hand-touched surfaces of four post-handwashing sites, including faucets, paper dispensers, hand dryers, and exit door handles, were sampled (Fig. 1A). All accessible surfaces of faucets and exit door handles were swabbed (Fig. 1B). For paper dispensers, only the paper towel outlet surface (bottom part) was sampled. For hand dryers, both the air outlet and hand-receiver surfaces (if any) were swabbed. Each surface was wiped with vertical, diagonal, and horizontal S-strokes for 3 min. For each round of sampling, metadata describing the sample collection dates, daily temperature, relative humidity, and weather conditions were recorded in Supplementary Table S2. After sample collection, Polywipe was stored at 4 °C before returning to the laboratory for processing on the same day of sampling. Aseptically, 45 mL of 1% peptone water-tween 80 was added to the stomacher bag containing Polywipe. The mixture was then put into a stomacher and run for 5 min, set at 3.8 rpm/s, followed by centrifugation at 4347 rpm for 15 min to separate microorganisms. After that, the pellet was resuspended with 1 mL sterile PBS. A total of 100 µL of the resuspension was used for enumeration of total bacterial counts (see Methods in "Total bacterial counts" section) and MDRO isolation (see Methods in "MDRO isolation" section), respectively. The remaining resuspension (~ 800 µL) was pelleted by centrifugation at 5000 rpm for 5 min, stored at − 80 °C, and subsequently processed as a direct environmental sample using metagenomic approach. Two negative controls, air and surface control, were prepared by holding Polywipe in the air and wiping down the laboratory bench surface for 3 min, respectively. They were then processed alongside washroom samples.

Total bacterial counts

Total bacterial counts were quantified using the aerobic plate count (APC) method. A 100 µL aliquot of the sample resuspension from Methods "Sample collection and processing" section was spread evenly onto brain heart infusion (BHI) agar (Becton Dickinson, USA) using a sterile L-shaped disposable spreader. All plates were incubated aerobically at 37 °C for 48 h. Colony-forming units (CFU) were enumerated using the DigiCounter™ of the Biomic V3 Microbiology System (Giles Scientific Inc., Santa Barbara, CA, USA). Due to the irregular shapes of the sampled surfaces, which preclude accurate surface area measurements, bioburden was expressed as total CFU per site. To account for high variability in the data, the geometric mean of total CFU was calculated for each post-handwashing site.

MDRO isolation

A total of 100 µL of the resuspension from Methods "Sample collection and processing" section was inoculated into buffered peptone water (BPW; Thermo Fisher Scientific, USA) for the isolation of MDRO. After incubation at 37 °C overnight, 100 µL of BPW was sub-cultured into brain heart infusion (BHI) broth (Becton Dickinson, USA) supplemented with antibiotics according to Supplementary Table S11, followed by incubation at 37 °C overnight. Subsequently, incubated antibiotic-BHI broth was plated out onto five chromogenic agars for further selection: CHROMagar ESBL, MRSA, mSuperCARBA, VRE (CHROMagar, Paris, France), and RAPID' *P. aeruginosa* Agar (Bio-Rad Laboratories, Hercules, CA) (Supplementary Table S12). All plates were then incubated at 37 °C for 48 h, followed by examination for the colors and morphologies of the colonies as instructed by the manufacturer. MALDI-TOF was performed to confirm the identity of the colonies using the MALDI Biotyper sirius System (Bruker Daltonics, Bremen, Germany) and MBT Compass HT software (v5.1.300) with MBT Compass Library (v12.0.0.0). Isolates with a score > 2.00 were selected and subjected to subsequent antibiotic susceptibility testing.

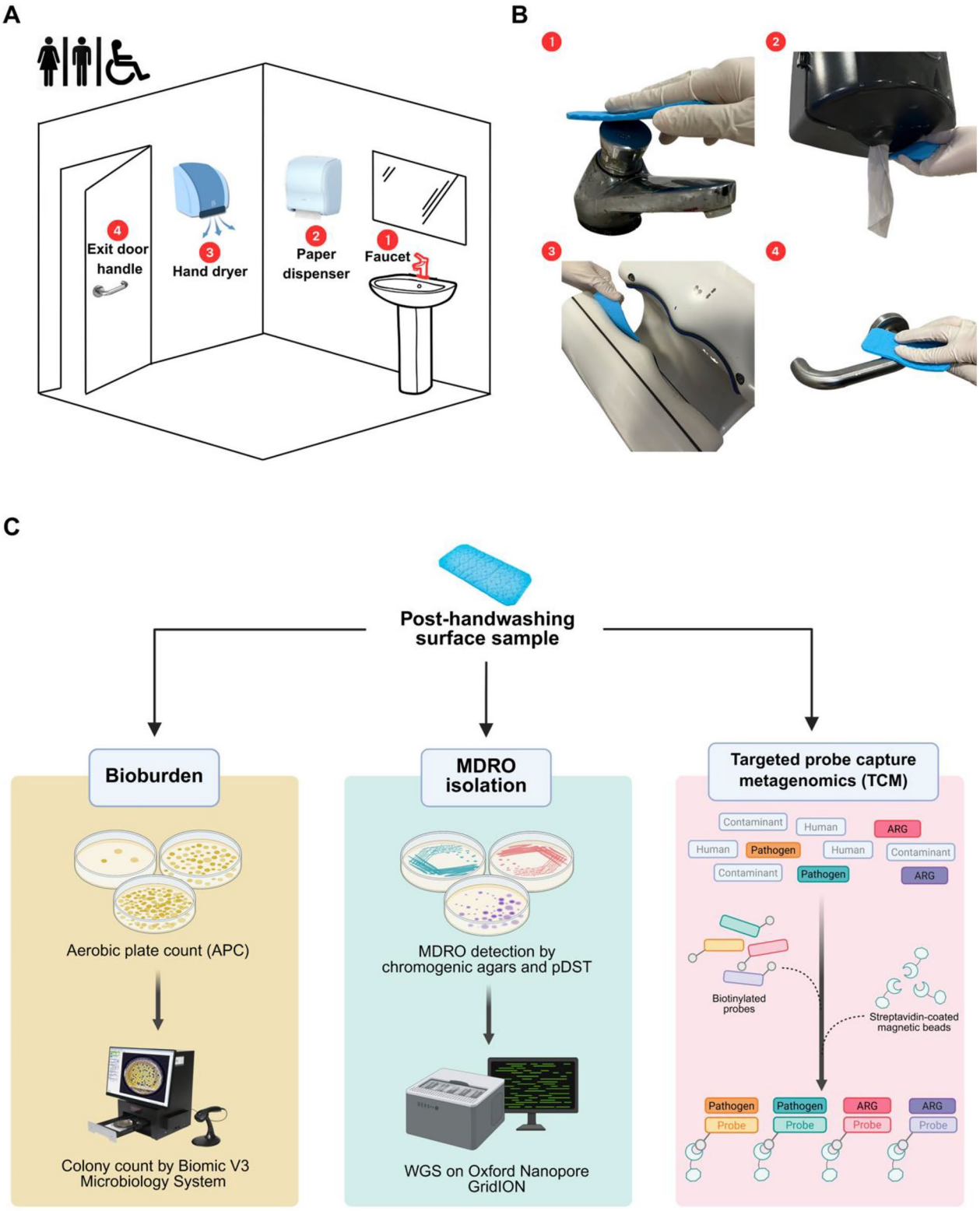

Fig. 1 Study design and analysis workflow of the MDRO and ARG surveillance of post-handwashing areas in public washrooms. **A** Four post-handwashing sites in public washrooms, including faucets, paper dispensers, hand dryers, and exit door handles, with high risk of contamination by MDROs and ARGs, were sampled. **B** Photographs showing the surfaces of each post-handwashing site being sampled. **C** Diagram showing the three analysis workflows for all surface samples. From left to right, the total bacterial count of each sample was enumerated using the aerobic plate count method; MDROs were isolated from surface samples using chromogenic agars, followed by confirmation of drug resistance status by pDST; Targeted probe capture enrichment was performed on metagenomic libraries to enrich pathogen and ARG reads. *MDRO* multidrug-resistant organisms, *pDST* phenotypic drug sensitivity testing, *ARG* antimicrobial resistance gene, *WGS* whole genome sequencing

Phenotypic Drug Susceptibility Testing (pDST) was performed on isolated bacterial colonies to confirm their drug resistance profile according to the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol [33]. For each bacterial isolate, antibiotic discs were placed on Mueller–Hinton agar (MHA; Becton Dickinson, USA). To classify bacterial isolates as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR), antibiotic panels for *S. aureus*, *Pseudomonas aeruginosa*, and members of the *Enterobacterales* family were selected based on Magiorakos et al. [34] (Supplementary Table S13A–C). Zones of inhibition were measured by the Biomic V3 Microbiology System (Giles Scientific Inc., Santa Barbara, CA). The status of resistance or susceptibility was determined according to Performance Standards for Antimicrobial Susceptibility Testing, CLSI M100 (35th ed) [35]. Specifically, methicillin-resistant *S. aureus* (MRSA) was identified by resistance to cefoxitin (30 µg), with an inhibition zone diameter ≤ 21 mm. Carbapenem-resistant *P. aeruginosa* (CRPA) was defined by resistance to at least one carbapenem drug (imipenem, doripenem, or meropenem), with an inhibition zone diameter ≤ 15 mm. Extended-spectrum beta-lactamase (ESBL) production was confirmed using the combined disk method, testing cefotaxime (CTX, 30 µg) alone and with clavulanic acid (CTX/CLA, 30/10 µg), as well as cefuroxime (CXM, 30 µg) alone and with clavulanic acid (CXM/CLA, 30/10 µg). An isolate was classified as ESBL-positive if the inhibition zone diameter of either combined disk (CTX/CLA or CXM/CLA) was ≥ 5 mm larger than that of the corresponding single disk (CTX or CXM).

DNA extraction

DNA extraction was performed on MDRO isolates and direct environmental samples using QIAamp BiOstic Bacteremia DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. In brief, DNA was released after bacterial cell lysis via both chemical and mechanical homogenization. DNA was subsequently purified by column-based method and collected by elution buffer. The DNA concentration was determined by Qubit® fluorometer 4.0 (Thermo Fisher Scientific, USA) with Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, USA).

Whole genome sequencing of MDRO isolate by Oxford nanopore

For cultured MDRO isolates, libraries were constructed using the Rapid Barcoding Kit (SQK-RBK 114.96) from Oxford Nanopore Technologies (ONT) according to the manufacturer's protocol. In brief, 200 ng of extracted DNA were fragmented and barcoded simultaneously by transposonase. A total of 16 barcoded libraries were pooled in equal volumes, cleaned with AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA), and followed by adapter ligation. Subsequently, sequencing was performed using R10.4.1 flow cells on GridION for 72 h and basecalled using the super accurate (SUP) model. Raw Nanopore reads were then de novo assembled using Hybracter (v0.11.2) [36]. The assembly was then processed by Bactopia (v3.2.0) [37] for the complete analysis of the bacterial genome. Species identification by GTDB-Tk [38, 39], in silico multilocus sequence typing (MLST) by mlst [40, 41], and ARG profiles by AMRFinderPlus [42] were obtained based on the assembly.

Metagenomic library construction and targeted probe capture enrichment

Metagenomic libraries were constructed using the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (NEB, UK) according to the manufacturer's protocol, with modification of adaptor concentration adjusted to 0.3 µM to avoid adaptor dimers. Quantification of library concentration was performed using Qubit® fluorometer 4.0 (Thermo Fisher Scientific, USA) with Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). Quality of libraries was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) in combination with Agilent High Sensitivity DNA Kit (Agilent Technologies, Palo Alto, CA, USA). Targeted probe capture was then performed on metagenomic libraries using the Respiratory Pathogen ID/AMR Enrichment Panel (RPIP) Kit (Illumina, San Diego, CA, USA). Overnight hybridization was performed following the manufacturer's protocol. The captured metagenomic libraries were then sequenced on Illumina Novaseq 6000 (Illumina, San Diego, CA, USA) using paired-end 150-bp reads. Two GB of data per captured metagenomic library was generated.

Bioinformatic analysis

Raw sequencing reads were analyzed by Explify® RPIP Data Analysis (v2.1.1). A collection of third-party bioinformatic tools was also used to verify the results obtained by Explify (Supplementary Fig. S1). Raw fastq files of paired-end reads were analyzed by Explify RPIP Data Analysis following the manufacturer's instructions. Reads with reads per kilobase million (RPKM) less than 5 were excluded from subsequent analysis.

For third-party bioinformatics analysis, raw sequencing reads were filtered for low-quality bases with Phred score < 33 and trimmed for adaptors using fastp (v0.23.4) [43]. Human reads removal was then performed by mapping filtered and adaptor-trimmed reads to the human reference genome assembly GRCh38 (accession GCA_000001405.29) using bowtie2 (v2.5.3) [44] and samtools (v1.17) [45]; all mapped reads were removed.

Human-free reads were then subjected to taxonomic profiling by two third-party taxonomic classifiers, Kraken2/Bracken (v2.1.3) [46] and MetaPhlan4 (v4.1.1) [47]. Species with a relative abundance of less than 0.1% were excluded from analysis. Similarly, two ARG profiling tools, ResFinder (v 4.5.0) [48] and DeepARG (1.0.4-0) [49], were used. For ResFinder, only ARG hits with ≥ 90% sequence identity and ≥ 60% coverage of reference sequence were included in subsequent analysis. For DeepARG, ARG hits with a minimum probability ≥ 80% and sequence coverage ≥ 80% were included. All contaminating microbial taxa and ARGs were statistically identified by the R package "decontam" (v1.22.0) [50] by comparing post-handwashing surface samples and the two controls (i.e., air and surface control), and were then excluded from subsequent analysis.

Calculation of MASH distances between metagenomic samples

Human commensal microbiome samples from gut [51], skin [52], respiratory tract [53], and urinary tract [54] were retrieved from the NCBI Sequence Read Archive (SRA) database. MASH sketches and MASH distances of all metagenomes were constructed and calculated using MASH (v2.3) [55]. In brief, MASH uses MinHash sketching to estimate the similarity between metagenomes by approximating the proportion of shared *k*-mers. The resulting MASH distance ranged from 0 to 1, with higher values indicating greater dissimilarity.

Alpha diversity and beta diversity

For measuring α diversity, species richness was calculated using the vegan package in R [56]. For β diversity, the Bray–Curtis dissimilarity matrix was determined using the vegdist function in the vegan package in R [57] and visualized using principal coordinate analysis (PCoA) by the prcomp package [58].

Statistical analysis, graphs and phylogenetic tree construction

Bar plots and heatmaps were constructed using RStudio (v2023.06.0) with the ggplot2 package [59]. Phylogenetic tree was constructed using Mashtree (v1.4.6) [60] and visualized using iTOL (v6.9.1) [61]. Kruskal–Wallis test was carried out for all non-parametric data. For all statistical comparisons, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were used as the significance levels.

Data availability

Raw sequence data generated are available in the NCBI Sequence Read Archive (SRA) database under BioProject accession PRJNA1250189.

Results

Overview of MDRO and AMR surveillance in post-handwashing areas

We collected a total of 232 surface samples from high-contact post-handwashing sites in public washrooms. For each washroom, four post-handwashing sites, including faucets ($n = 72$), paper dispensers ($n = 63$), hand dryers ($n = 55$), and exit door handles ($n = 42$), were sampled (Fig. 1A, B; Supplementary Table S1). The average daily temperature was 28.56 ± 2.03 °C, and the relative humidity was 79.71 ± 7.09% (Supplementary Table S2).

Each surface sample was subjected to three analysis workflows (Fig. 1C). First, total bacterial counts were enumerated to assess the bioburden level using the aerobic plate count (APC) method. Second, the presence of MDRO was determined using chromogenic agars, followed by confirmation of drug resistance status by phenotypic drug sensitivity testing (pDST). Third, targeted probe capture enrichment was performed on metagenomic libraries to enrich pathogen and clinically relevant ARG reads, followed by taxonomic classification and ARG profiling. This multi-modal approach enabled a comprehensive interrogation of pathogen and AMR within the post-handwashing area.

Metagenomic analyses were performed using the companion bioinformatic analysis platform, Explify RPIP (Supplementary Fig. S1). A collection of third-party bioinformatic tools was also employed to validate Explify RPIP results (Supplementary Fig. S1). Of the initial 232 samples, 28 were excluded from downstream analysis due to failed library construction, resulting in 204 samples for subsequent analysis.

Bioburden of the post-handwashing areas

Bioburden of the post-handwashing sites was assessed using the APC method. Despite the highly variable bacterial counts observed across all sites (Fig. 2), statistical analysis revealed significant differences among the four sites ($p = 0.00107$, Kruskal–Wallis test). Faucets had the highest microbial load, with an average colony-forming units (CFU) of 1.6×10^5, likely due to their moist microenvironments that promote microbial proliferation. Hand dryers ranked second, harboring an average of 7.7×10^4 CFU, followed by exit door handles (6.4×10^4 CFU), while paper dispensers had the lowest bioburden (5.8×10^4 CFU). These findings revealed varying bioburden levels across different post-handwashing sites, highlighting the need for targeted cleaning protocols.

Taxonomic profiling of the post-handwashing areas

High-throughput sequencing (HTS) of TCM libraries generated an average of 18,443,657 ± 10,066,472 reads per sample, with microbial reads comprising 79.71% ± 2.36%

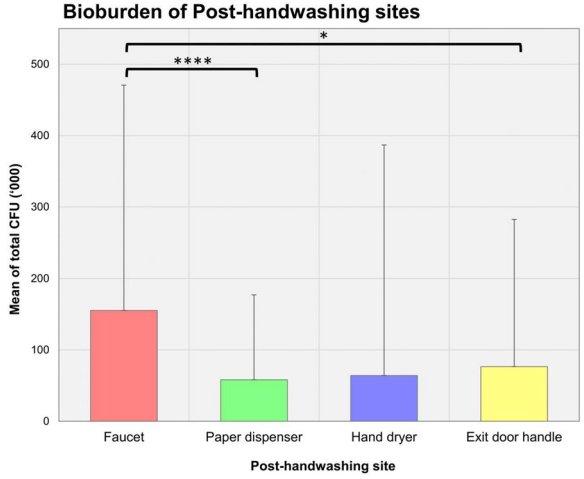

Fig. 2 Bioburden of post-handwashing sites in public washrooms. The bioburden of the four post-handwashing sites was assessed by aerobic plate count (APC). The mean of the total CFU of each site was calculated. Statistical significance: *$P < 0.05$, ****$P < 0.0001$. *CFU* colony-forming unit

of total reads (Supplementary Fig. S2A). Human-derived reads and unclassified reads accounted for 7.25% ± 1.82% and 7.21% ± 0.85% of total reads, respectively. Taxonomic classification of microbial reads revealed 97.17% ± 0.22% (14,546,045 ± 2,474,831 reads) as bacteria, 2.30% ± 0.15% (341,272 ± 35,932 reads) as fungi, and 0.53% ± 0.09% (78,669 ± 8324 reads) as viruses (Supplementary Figure S2B).

Alpha diversity, measured by species richness, indicated that hand dryers represented the most taxonomically diverse surfaces among post-handwashing sites (Fig. 3A), although the inter-site differences were statistically insignificant ($p > 0.05$, Kruskal–Wallis test). Beta diversity, visualized by principal coordinates analysis (PCoA), showed that faucet microbiomes display the greatest dissimilarity (i.e., the greatest cluster area of 0.471), followed by paper dispensers (cluster area = 0.428) (Fig. 3B). Hand dryers displayed the smallest dissimilarity (cluster area = 0.324), indicating they share a similar microbiome composition despite being sampled from different washrooms.

The top 20 dominant species of each post-handwashing site, ranked by average reads per kilobase million (RPKM), were identified (Fig. 3C). Five bacterial species, including *Moraxella osloensis*, *Rothia mucilaginosa*, *Actinomyces naeslundii*, *Pseudomonas aeruginosa*, and *Pseudomonas stutzeri*, were ubiquitous across all sites. Site-specific taxonomic patterns were observed. Seven taxa were exclusively detected on faucet surfaces, including *Acinetobacter nosocomialis, Acinetobacter israelii*, *Actinomyces odontolyticus*, *Corynebacterium striatum*, *Mycobacterium gordonae*, *Stenotrophomonas maltophilia*, and *Streptococcus constellatus*. Paper dispensers uniquely detected *Enterococcus faecium*, *Eubacterium brachy*, *Fusarium solani*, *Neisseria flavescens*, *Slackia exigua*, and *Tsukamurella pulmonis*. Hand dryers exclusively detected with *Delftia acidovorans*, *Enterococcus faecalis*, *Klebsiella quasipneumoniae*, and *Streptococcus intermedius*, whereas *Acinetobacter baumannii*, *E. coli*, *Finegoldia magna*, Herpes Simplex virus 1 (HSV-1), and *Moraxella catarrhalis* were only detected in exit door handles. Notably, taxa associated with clinically important species from the WHO Bacterial Priority Pathogen List (2024) were detected. Among the four post-handwashing sites, exit door handles detected the highest number of critical priority taxa ($n = 5$), including *A. baumannii*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *S. aureus*, highlighting their potential role as reservoirs for pathogens.

To validate the taxonomic assignments by Explify RPIP (Supplementary Table S3), taxonomic classification by two third-party taxonomic classifiers, Kraken2 and MetaPhlAn4, was performed (Supplementary Tables S4 and S5). Concordance analysis revealed partial overlap (42.41% ± 10.59%) between species detected by Explify RPIP and third-party classifiers (Supplementary Table S6). These discrepancies likely arise from differences in classification algorithms and reference databases used by different classifiers [62–65].

Since human-associated microbiota are major contributors to microbial communities of the post-handwashing surfaces [4, 66], we next assessed the similarity of post-handwashing microbiomes to human commensal microbiomes by determining the MASH (Minhash-based) distances, which approximate the genomic dissimilarity between microbial communities. To investigate this, we randomly selected forty publicly available metagenomes from four commensal microbiome sites, including the human gut [51], skin [52], respiratory tract [53], and urinary tract [54] (i.e., 10 samples per site). Mean MASH distances between post-handwashing sites and human commensal microbiomes were calculated (Table 1). Faucet microbiomes exhibited the lowest MASH distances to human skin and gut microbiomes, indicating their microbial composition is akin to these commensal microbiomes. In contrast, all post-handwashing microbiomes displayed higher MASH distances than controls to respiratory tract and urinary tract microbiomes, suggesting dissimilarity to these two commensal microbiomes.

ARG profiling of the post-handwashing areas

To characterize the AMR determinants in the post-handwashing areas, we performed ARG profiling for all surface samples. ARGs conferring resistance to macrolide–lincosamide–streptogramin (MLS) dominated the post-handwashing area resistome, accounting for 29.1% of total ARG hits, followed by aminoglycoside (28.1%) and beta-lactam (21.5%) resistance genes (Fig. 4A).

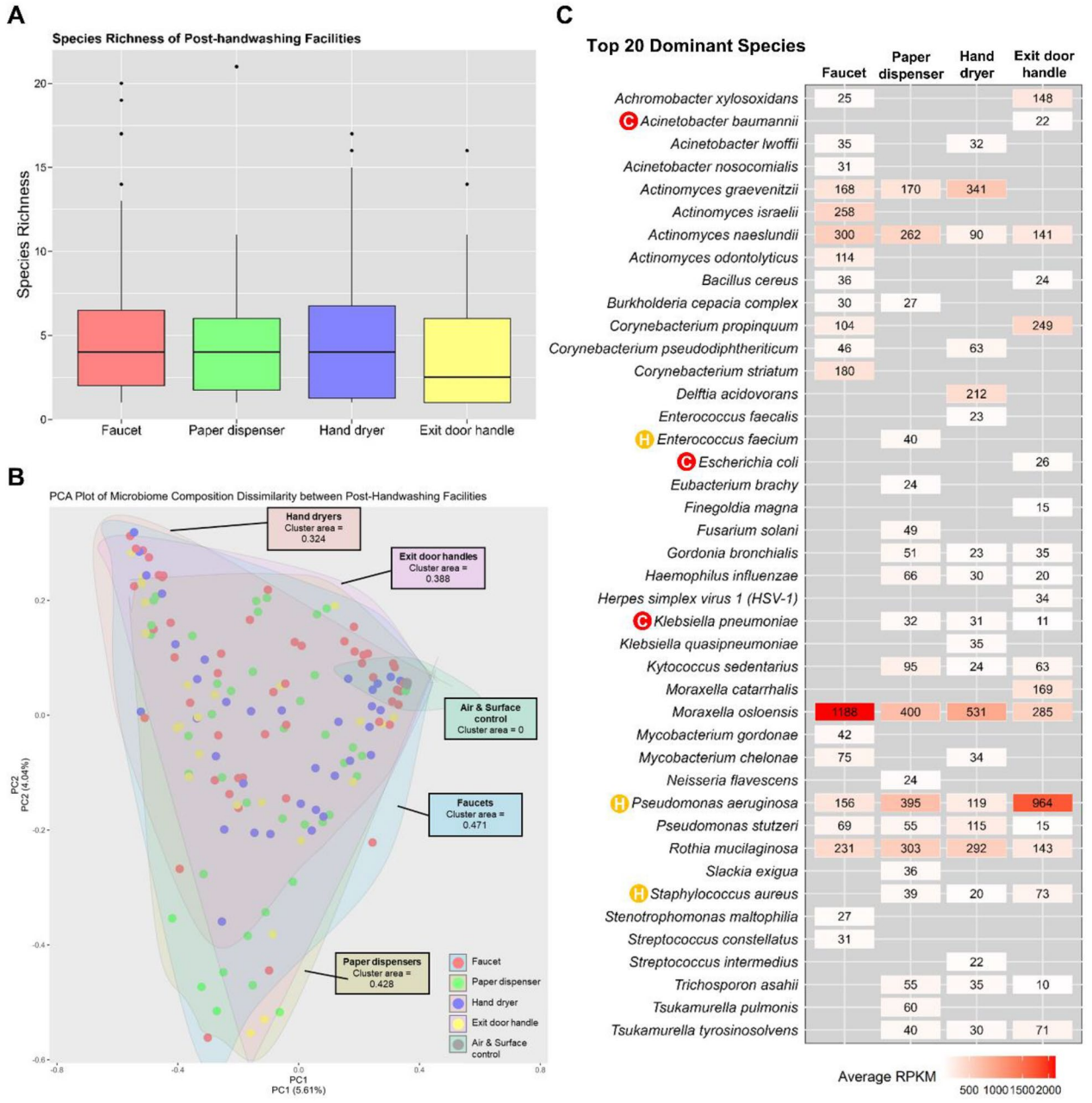

Fig. 3 Targeted probe capture metagenomics (TCM)-enabled microbiome analysis of the post-handwashing area in public washrooms. **A** Bar plot showing the species richness of each post-handwashing site. **B** Principal coordinates analysis (PCoA) showing microbiome composition dissimilarity of post-handwashing sites in different washrooms. **C** The top 20 dominant species of each post-handwashing site identified by TCM, ranked by average RPKM of each species detected. Red and orange circles denote species classified as "Critical" and "High" priority groups in the WHO Bacterial Priority Pathogen List (2024) [67], respectively. *RPKM*, reads per kilobase million

Despite the faucet exhibiting the highest ARG diversity ($n = 91$), it yielded the lowest average AMR-associated reads (Fig. 4B). Hierarchical clustering of the 20 most abundant ARGs per site, ranked by average RPKM, revealed a closer clustering between paper dispenser and exit door handle (Fig. 4C), suggesting their similarity in resistome compositions. Of clinical importance, *mecA*, the genetic determinant for MRSA, was enriched in exit door handles. Together with the enrichment of *S. aureus* observed (Fig. 3C), these findings suggest that exit door handles may serve as potential reservoirs for MRSA.

ARG identification by Explify RPIP (Supplementary Table S7) was validated by two third-party ARG profiling tools, ResFinder and DeepARG (Supplementary Tables S8 and S9). Concordance analysis confirmed a

Table 1 Mean MASH distances between human commensal microbiomes and post-handwashing sites

Post-handwashing sites	Skin	Gut	Respiratory tract	Urinary tract
Faucet	0.18 ± 0.05	0.39 ± 0.29	0.21 ± 0.10	0.58 ± 0.36
Paper dispenser	0.19 ± 0.13	0.44 ± 0.32	0.18 ± 0.11	0.54 ± 0.36
Hand dryer	0.18 ± 0.08	0.44 ± 0.32	0.18 ± 0.07	0.57 ± 0.36
Exit door handles	0.19 ± 0.10	0.48 ± 0.34	0.17 ± 0.03	0.55 ± 0.36
Controls	0.21 ± 0.03	0.68 ± 0.37	0.17 ± 0.02	0.47 ± 0.32

Means ± standard deviations of MASH distances between microbiomes of the post-handwashing sites and human commensal microbiomes. MASH distances between laboratory environmental controls (i.e., air and surface controls) and human commensal microbiomes were also calculated as a baseline for comparison

substantial proportion of ARGs (72.86% ± 4.88%) overlapped between them (Supplementary Table S10).

Isolation of clinically significant MDRO strains from post-handwashing areas

To assess the viability and prevalence of MDROs, we looked for the presence of five clinically critical pathogens listed in the WHO Bacterial Priority Pathogen List 2024 [67] using chromogenic agars. The five target MDROs were extended-spectrum beta-lactamase-producing *Enterobacterales* (ESBL-E), carbapenem-resistant *Enterobacterales* (CRE), vancomycin-resistant *Enterococcus* (VRE), CRPA, and MRSA (Supplementary Table S12). Among the 232 surface samples, 17 MDRO isolates (7.33%) were identified (Fig. 5A; Supplementary Table S14A–C). ESBL-E was predominant, comprising 59% of isolates, followed by CRPA (29%) and MRSA (12%) (Fig. 5B). No CRE or VRE was isolated. Among the post-handwashing sites, faucets represented the most contaminated sites by MDRO, harboring 53% of isolates, followed by hand dryers (24%), exit door handles (18%), and paper dispensers (6%, Fig. 5C). All MDRO isolates were classified as MDR, no XDR or PDR strains were identified.

Whole-genome sequencing (WGS) and in silico multilocus sequence typing (MLST) of MDRO isolates identified clinically significant sequence types (STs) and their full drug resistance profiles. Two community-acquired MRSA (CA-MRSA) clones were isolated from post-handwashing areas (Fig. 5A; Supplementary Table S14A). One of the clones, recovered from a paper dispenser, was assigned to ST338, carrying the staphylococcal cassette chromosome *mec* (*SCCmec*) type Vb, *spa*-type t437, and *agr*-type I. The other clone recovered from the hand dryer was assigned to ST1457, typed with SCC*mec* IV, *spa*-type t067, and *agr*-type II. Both clones possessed *mecA*, consistent with their phenotypic resistance to cefoxitin (Supplementary Table S14A). ST338 was previously reported as a multidrug-resistant, highly virulent CA-MRSA clone identified in Taiwan, China, and Poland [68–72], and was linked to severe bloodstream infections. Consistent with previous studies, this clone also carried *ermB*, which confers resistance to MLS antibiotics [70, 72]. In contrast, ST1457 is poorly documented, suggesting its limited clinical relevance. All five CRPA clones were recovered from faucets, spanning across four STs, including ST27, ST313, ST1182, and ST1341 (Fig. 5A; Supplementary Table S14B). Among them, ST313 is the most well-documented. Although reported as an intestinal colonizer of healthy individuals [73], this clone is commonly associated with urinary tract infections (UTIs) [74]. Consistent with our pDST results, ST313 was previously reported to be resistant to carbapenems [75]. ST27 and ST1182 have been detected in clinical samples and drinking water networks [76–78], whereas ST1341 is less characterized. Among the ten ESBL-E isolates, two high-risk STs were identified (Fig. 5A; Supplementary Table S14C). The *Enterobacter hormaechei* ST133 clone, recovered from a hand dryer, was reported as a virulent and MDR strain linked to bloodstream infection [79, 80]. However, the clone we discovered lacked critical beta-lactamase genes (e.g., bla*CTX-M*, bla*NDM*) as previously reported [79, 80], and was tested sensitive to carbapenems. Additionally, a multidrug-resistant *E. coli* ST1193 clone of serotype O75:H5 from a faucet sample was positive for bla*CTX-M-55* (Supplementary Table S14C). This clone was previously reported as a globally emerging clone and was associated with community-onset UTIs and bloodstream infections [81–83]. Collectively, the isolation of these clinically important STs together with their drug-resistant traits further highlights the post-handwashing areas as potential hubs for disseminating virulent and drug-resistant strains in the washroom environments.

Targeted probe capture enrichment identified opportunistic pathogens and clinically critical ARGs in post-handwashing areas

We next determined the presence of human pathogenic taxa, which were defined according to the American Biological Safety Association (ABSA) Risk Group Database with Risk Group 2 or above [84]. Using TCM, we identified a total of 47 species of opportunistic pathogens across all post-handwashing sites (Fig. 6A). TCM revealed that 65.2% of the surface samples were detected with at least one of these opportunistic pathogens. Among the four sites, faucets harbored the greatest number of species ($n = 33$), followed by paper dispensers ($n = 29$), hand dryers ($n = 24$), and exit door handles ($n = 19$). Clinically important opportunistic pathogens were enriched on specific sites. Two important environmental species often associated with nosocomial infections, *A. baumannii* and *P. aeruginosa*, were predominantly enriched in faucets and exit door handles, respectively [85]. *Enterococcus faecalis* and *Enterococcus faecium*, two *Enterococcus* spp. of clinical importance associated with UTIs, wound

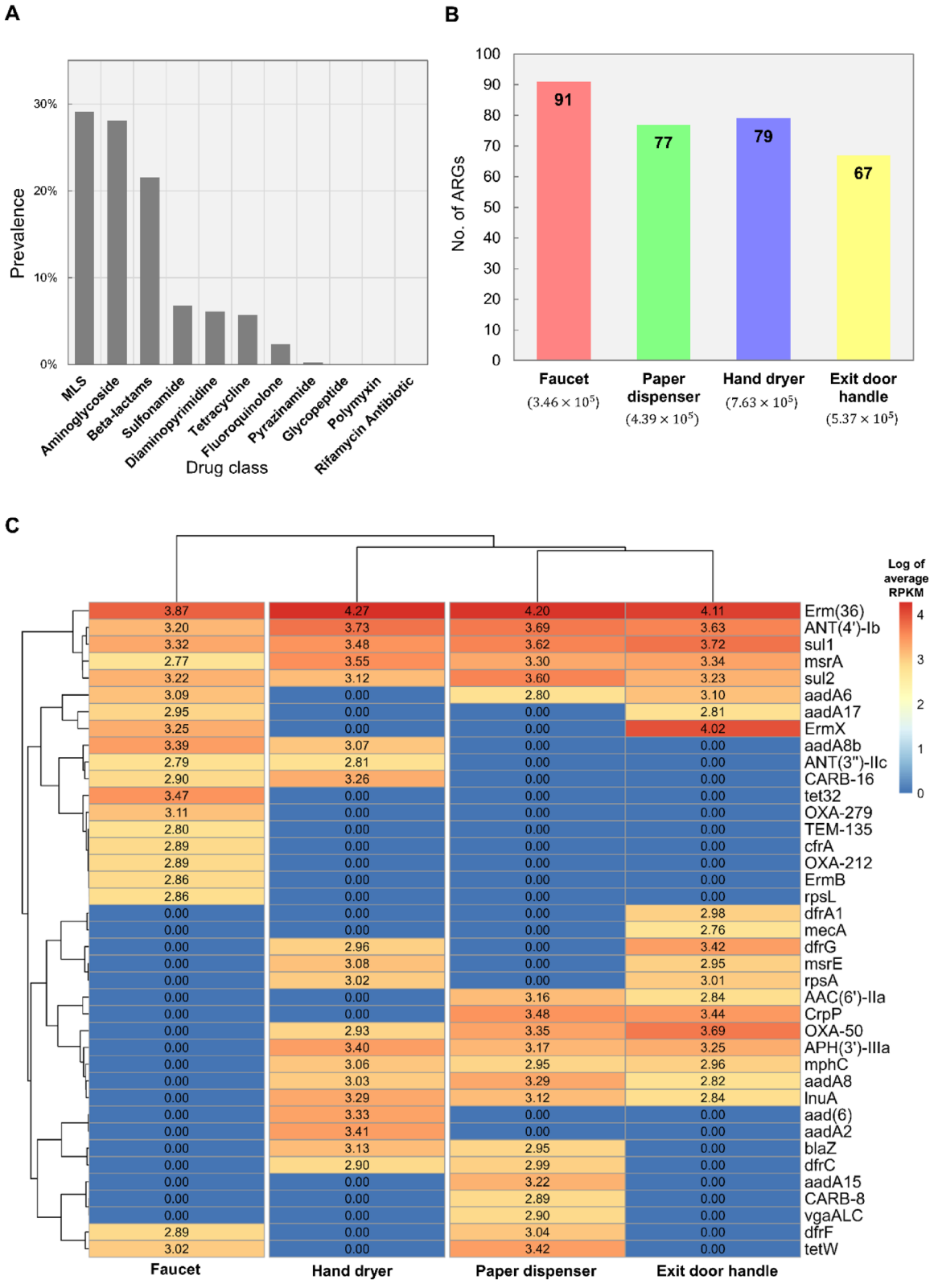

Fig. 4 Targeted probe capture metagenomics (TCM)-enabled ARG profiling of the post-handwashing area in public washrooms. **A** Total percentage of reads assigned to ARGs of different drug classes by TCM. **B** Total number of ARGs (bars) and total reads assigned to ARGs (in parentheses) of different post-handwashing sites. **C** Hierarchical clustering of the top 20 dominant ARGs identified by TCM at each post-handwashing site. *ARG* antimicrobial resistance gene, *RPKM* reads per kilobase million

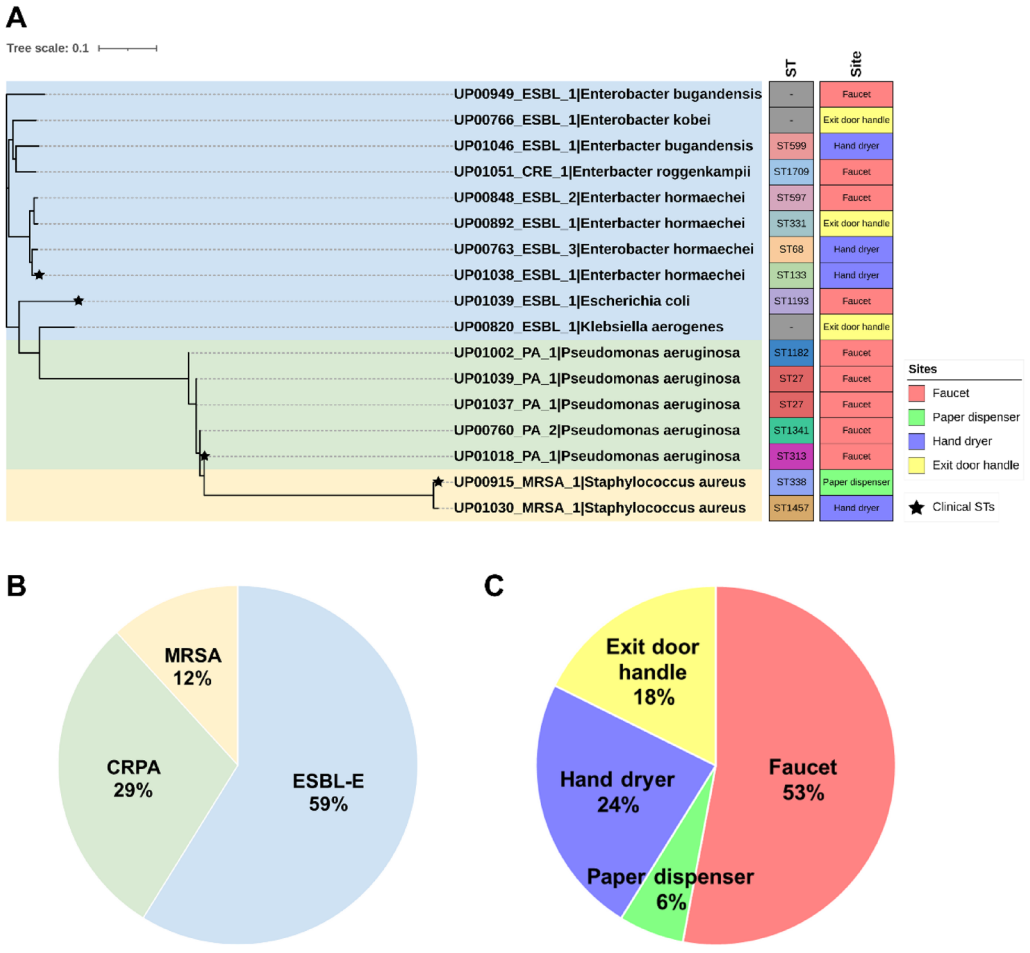

Fig. 5 Prevalence and distribution of MDROs identified from the post-handwashing areas of public washrooms. **A** The phylogenetic tree of all MDRO isolates recovered from the post-handwashing areas of public washrooms ($n = 17$). **B** The prevalence of MDRO isolates by species. **C** The distribution of MDRO isolates identified from different post-handwashing sites. *MDRO* multidrug-resistant organisms

infections, sepsis, and endocarditis [86], were enriched in hand dryers and paper dispensers, respectively. Common agents of community-acquired pneumonia, namely *K. pneumoniae* and *Streptococcus pneumoniae*, were detected in hand dryers, posing potential infection risks to washroom users [87]. Important skin infection agents, including *S. aureus* [88], *P. aeruginosa* [89], and *E. coli* [90], were enriched in exit door handles, alongside Herpes simplex virus 1 (HSV-1) [91]. Additionally, *S. agalactiae* (Group B *Streptococcus*), a critical pathogen in neonatal and immunocompromised infections [92], was exclusively detected in paper dispensers. The presence of these opportunistic and clinically relevant pathogens implicates an elevated infection risk of microbial transmission and potential infection in the post-handwashing areas.

To assess the AMR burden of the post-handwashing area, we specifically looked for the presence of clinically critical ARGs. A total of 13 high-risk ARGs were detected (Fig. 6B). At least one of these high-risk ARGs was detected in 63.7% of samples. Among the four sites, paper dispensers harbored the highest number ($n = 8$). *mecA* was detected in all sites, with the highest number of average reads detected from exit door handles. In addition, bla*GES-5* was also detected across all sites, with faucets harboring the highest average reads. Site-specific identification of high-risk ARGs was also observed. For instance, bla*SHV-104* and *vanC* were exclusively detected in faucets, while carbapenemases bla*IMP-26* and bla*NDM-4* were unique to hand dryers. Six high-risk beta-lactamase genes were exclusively found in paper dispensers, including bla*CTX-M-64*, bla*CTX-M-65*, bla*GES-15*, bla*NDM-5*, bla*SHV-1*, and bla*SHV-11*. All the high-risk ARGs detected by Explify RPIP were concurrently detected by third-party ARG profiling tools, authenticating their presence on post-handwashing surfaces (Supplementary Fig. S3). These findings further demonstrate the pervasiveness of clinically significant resistance determinants in high-contact washroom spaces, warranting the need

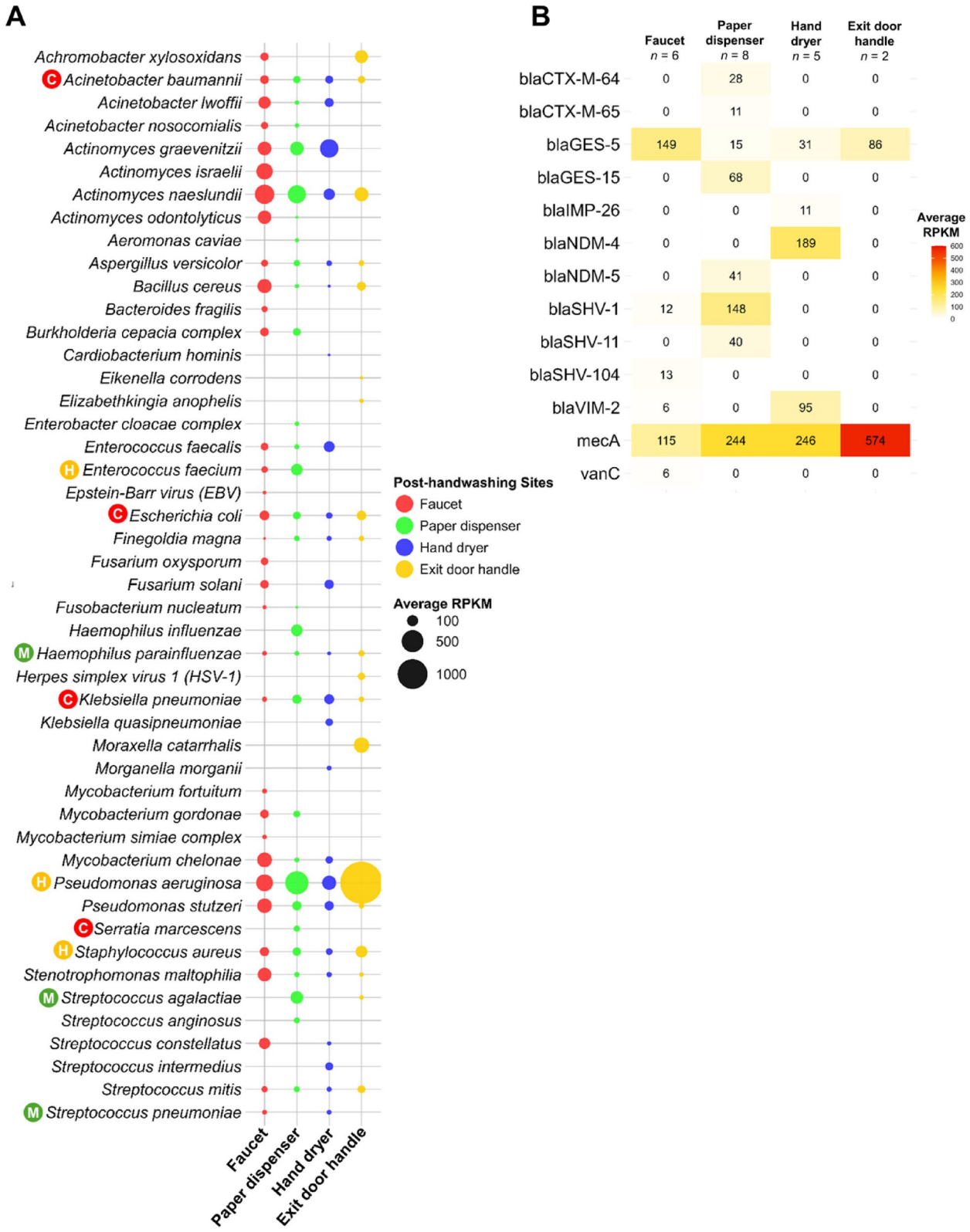

Fig. 6 Health impact of microbial taxa and ARGs identified from the post-handwashing areas of public washrooms. **A** Average RPKM assigned to microbial taxa with Risk Group 2 or above in the American Biological Safety Association's Risk Group Database [84] at different post-handwashing sites. Red, orange, and green circles denote species classified as "Critical", "High", and "Medium" priority groups in the WHO Bacterial Priority Pathogen List (2024) [67], respectively. **B** Heatmap showing the average RPKM assigned to clinically important ARGs at each site. *ARG* antimicrobial resistance gene, *RPKM* reads per kilobase million

for enhanced hygiene practices to mitigate AMR dissemination risks.

Discussion

Over the past decades, the rise of AMR has represented one of the most pressing public health threats, and it is exacerbated by urbanization and various anthropogenic activities [93–95]. In response, the One-Health initiative [96], a global public health collaborative framework addressing AMR from human, animal, and environmental aspects, has been advocated. While most surveillance efforts have been focusing on healthcare and agriculture contexts, environmental surveillance, particularly the built environments, is often neglected when it comes to AMR surveillance [97–99]. This study, therefore, addresses this gap by characterizing MDROs and ARGs in public washrooms, which are high-traffic urban hubs implicated in pathogen and antimicrobial determinants transmission [2–4]. We are particularly interested in the role of the post-handwashing area, a key junction where re-contamination of washed hands might occur, as a reservoir for pathogen and AMR transmission. Herein, we collected 232 surface samples from four high-contact post-handwashing sites of public washrooms across Hong Kong (Fig. 1A, B; Supplementary Table S1). Our integrated approach, combining conventional culture-based methods and the novel TCM approach, revealed this area as a reservoir for opportunistic pathogens and clinically critical ARGs. Culture-based methods confirmed the presence of viable MDROs, including MRSA, ESBL-E, and CRPA in the post-handwashing area (Fig. 5; Supplementary Table S14A-C), while TCM detected the presence of low-abundance pathogens and clinically critical ARGs from direct environmental samples (Fig. 6).

Our results suggest that the post-handwashing areas of public washrooms might be potential hotspots of MRSA infection. Even though only two viable MRSA isolates were identified from the culture-based approach (Fig. 5; Supplementary Table S14A), taxonomic and ARG profiling revealed the concurrent enrichment of *S. aureus* and *mecA* reads in exit door handles of public washrooms, implicating that these sites could be an important reservoir of MRSA (Figs. 3C and 6B). This finding is consistent with the 2019 study by Suen et al. [2], who also reported the enrichment of *S. aureus* on exit door handles, suggesting the persistence of *S. aureus* at this post-handwashing site. WGS of MRSA isolates identified a clinically significant clone, *ermB*-positive ST338-SCC*mec* Vb, which was linked to severe bloodstream infection cases [70, 71]. Taken together, we propose that exit door handles of public washrooms may serve as a critical hotspot for community MRSA dissemination via skin contact, warranting targeted disinfection procedures at these sites.

Taxonomic profiling revealed the dominance of human-associated commensals and environmental strains at post-handwashing areas (Fig. 3C). While none of these taxa are obligate pathogens, their roles as opportunistic pathogens in healthcare and community settings were well characterized. For instance, *A. baumannii*, detected as one of the dominating species from exit door handles (Fig. 3C), is a leading cause of ventilator-associated pneumonia (VAPs) and frequently harbors carbapenemase genes [100]. Although no carbapenem-resistant strains were recovered in this study, they were previously identified from faucets, hand dryers, and air samples of public washrooms [101, 102]. Similarly, we identified *E. coli* [103, 104], *K. pneumoniae* [105–107], *P. aeruginosa* [108, 109], and *Enterococcus* spp. [21], all of which have been previously reported to exhibit antimicrobial resistance in washroom environments. MRSA has also been extensively reported from public washrooms [110, 111], with multiple studies reporting its isolation from various surfaces, including toilet seats [8, 112], exit door handles [113], and floors [21]. In addition to previously reported opportunistic pathogens, we further revealed a broader spectrum of opportunistic pathogens present in public washrooms, namely *Haemophilus influenzae*, *M. catarrhalis*, *S. agalactiae*, *S. pneumoniae*, and *Mycobacterium chelonae* (Fig. 6A). Together with the simultaneous detection of clinically important ARGs (Fig. 6B), these findings highlight public washrooms as a critical hub for opportunistic pathogens, and therefore the need for enhanced hygiene vigilance in public washrooms to prevent potential infections, especially for the immunocompromised cohorts [114].

This study illustrates the first application of TCM in a large-scale pathogen and AMR surveillance of the urban built environments. Our work highlights several key advantages of TCM's application in environmental surveillance. First, TCM was able to remove non-target reads from environmental samples. In this study, the proportion of human-associated reads was significantly lowered to 7.25% for all samples, whereas they can constitute up to 70% of total reads in an unbiased metagenomics workflow [115]. This reduction allows the detection of target reads at a much lower sequencing depth and thus faster turnaround time. Second, the proportion of unclassified reads was lowered to 7.21%, while it can constitute up to 40% in an unbiased workflow [115]. Third, TCM enables a robust detection of rare pathogens and clinically important ARGs in low-biomass environmental samples, demonstrating its utility in uncovering hidden AMR threats in built environments. While unbiased metagenomics workflows often require at least 200 million reads for comprehensive ARG profiling [116], our TCM workflow achieved similar detection at an average read depth of 18 million reads. This represents an 11-fold

reduction in sequencing depth requirements, further highlighting its cost-effectiveness for public health surveillance. These observations suggest that TCM has the potential to extend its applications beyond washroom environments to other built environments, such as hospitals, nursing homes, and schools, where pathogen surveillance is essential.

Despite the utility of TCM in uncovering hidden pathogenic and AMR transmission in public washrooms, this study has limitations. First, the nucleic acid extraction kit used in this study was designed to capture only DNA, thus precluding the detection of RNA viruses from subsequent metagenomic analysis. RNA viruses are etiological agents for numerous infectious diseases in humans and animals, posing significant public health problems globally [117]. These pathogens are frequently detected in washroom environments, where their presence may contribute to disease transmission [19, 118, 119]. Second, this study lacks genomic comparisons with clinical isolates from local hospitals or complementary epidemiology investigations. Therefore, we cannot assess the direct public health and clinical impacts of the MDROs identified. Such analyses are critical for determining whether the detected MDROs are genetically related to strains causing human infections or contribute to disease burden in the community. Third, TCM does not reflect the viability of the organisms, where the signal detected may come from deceased cells, leading to overestimation of pathogen load and thus infection risks in these environments. Fourth, the current probe panel used in the TCM approach was unable to discriminate between pathogenic and non-pathogenic strains within detected taxa, as it lacks strain-level resolution for virulence genes or pathogenic marker genes, thereby limiting clinical interpretability. Lastly, the cross-sectional design of this study captures a single time point, which may not fully reflect the dynamic nature of microbial contamination. Transient pathogens introduced into the washroom environment may be removed by routine cleaning protocols, potentially leading to an underestimation of the actual microbial load and diversity.

To address these limitations, future research should adopt a longitudinal approach to monitor microbial persistence in post-handwashing areas over time. Employing total nucleic acid extraction methods to capture both DNA and RNA will provide a more holistic view of the microbial and viral communities. Phylogenetic comparison with clinical isolates and epidemiological data will further elucidate the public health significance of the detected pathogens, enhancing the translational impact of future studies. Finally, customizable probe design incorporating pathogenic markers, paired with long-read sequencing, may enable strain-level characterization and species-resolved detection of microbial and ARG targets, respectively [24, 120, 121].

In summary, this study provides a comprehensive and nuanced characterization of the MDRO and AMR landscape of the post-handwashing areas in public washrooms using an integrated approach. By leveraging TCM, we uncovered a potential infection risk of MRSA via exit door handles as well as the presence of a broad assemblage of opportunistic pathogens and high-risk ARGs in public washrooms, highlighting the need for targeted infection control measures in these high-traffic environments. Our work demonstrates the utility of TCM in characterizing pathogen and AMR landscape from low-biomass environmental samples. As sequencing costs continue to decline and automation technologies advance (e.g., automated library automation) [122], we anticipate that TCM would revolutionize the toolbox for MDRO and AMR surveillance, ultimately leading to a paradigm shift in surveillance strategies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00806-2.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements
Not applicable.

Author contributions
AYTL: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, visualization, writing—original draft. CHL: Investigation, writing—review and editing. WYT: Investigation, writing—review and editing. CTMC: Investigation, methodology, writing—review and editing. TML: Investigation, writing—review and editing. LKPS: Funding acquisition, investigation, writing—review and editing. LKL: Formal analysis, writing—review and editing. EYYY: Investigation, writing—review and editing. TKL: Investigation, writing—review and editing. WKC: Investigation, writing—review and editing. MWC: Investigation, writing—review and editing. HSS: Investigation, writing—review and editing. FWNC: Investigation, writing—review and editing. SCL: Writing—review and editing. SNYS: Writing—review and editing. SKSY: Writing—review and editing. GKHS: Conceptualization, funding acquisition, investigation, project administration, resources, supervision, validation, writing—original draft. All authors read and approved the final manuscript.

Funding
This work was supported by Interdisciplinary Large External Project Application Scheme from the Faculty of Health and Social Sciences, The Hong Kong Polytechnic University (1-ZVZL) and the College Research Grant, Tung Wah College (PJC Code: 2023–00-51 CRG230402).

Data availability
Raw sequence data generated are available in the NCBI Sequence Read Archive (SRA) database under BioProject accession PRJNA1250189, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1250189.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 16 May 2025 / Accepted: 21 October 2025
Published online: 17 November 2025

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