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Research

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Abstract

Background: Genes make microorganisms resistant to antibiotics are overwhelmingly present in environments, and those in the air seem to consistently worsen human health, especially in built-in hospitals. In this study, we sampled two distinct seasonal solid-, liquid- and gaseous-state samples in a large occupied hospital, and mainly aim to study the temporal and spatial micro-ecology and antibiotic resistance gene (ARG) distribution patterns within hospital settings using culture-facilitated comparative metagenomics in combinations with other widely applied methods.

Results: Hospital aerosol resistome showed concentration of 0.00042 copies per 16S rRNA gene which was comparable to that of drinking water (0.00024 copies/16S rRNA). Winter aerosols showed higher resistome concentration and exhibited similar distribution patterns among different departments; while evident microbial accumulation and stronger level of inter-species ARG exchange was observed in summer aerosol samples. Network analysis and cultural isolate whole genome sequencing results confirmed some of these exchanges were mediated via plasmids. Additionally, ARGs and microbial community source tracking results illustrated Outpatient hall could serve as a major resistome pollution source all year long.

Conclusions: The state-of-art metagenomics analyses and culture facilitated

sequencing with strict cutoff guarantee the result accuracy of resistome pattern observed in built-in hospital environment, and our novel genotype sharing network confirmed the plasmid-mediated transferring mechanism in hospital air for the first time. Overall, this study illustrated valuable and quality hospital aerosol dissemination patterns and our effort will encourage more metagenomics applications in hospital studies.

Keywords: built-in hospital environment, aerosol, ARGs, culture-facilitated comparative metagenomics

Background

Antibiotics were invented to preventing infections and saving lives [1]. However, with the defensive mechanisms evolved from microorganisms, the genes make microorganisms resistant are “effectively weakening” and overwhelmingly present in environments after extensive applications worldwide [2,3]. Not only waterborne and soil related resistant microbes are threatening our health, but aerosol ARG pollution is a serious concern. Some research groups are exploring ARGs from the air we breathe, and those resistances seem to closely relate to air quality parameters. For instance, Li et al. [4] showed $PM_{2.5}$ possessed high concentration of ARG against β-lactam, quinolone, macrolide, tetracycline, sulfonamide, aminoglycoside and

66 vancomycin in China. Xie et al. [5] also noticed seasonal ARG variations
67 across industrial-urban-rural areas on an annual basis.

68 Humans are tethered to the built-in environment very closely. From the hospital
69 we are born in to the homes and offices we live and work in, the built-in
70 environment has become the most intimate ecosystem for humans. Lax et al.
71 [6–8] performed a wealth of work and provided valuable datasets related to the
72 indoor micro-ecology. They revealed human microbiota can influence indoor
73 microbial communities and also be shaped by built-in environment. Notably,
74 micro-ecology associated with hospital settings arguably has the most
75 profound implications because hospital-acquired infections (HAIs) have always
76 been considered as one of the main causes of patient deaths [7,9,10].
77 Therefore, lots of effort weighted on determining how microorganisms colonize,
78 persist, and change in the hospital indoor environment. Many previous studies
79 have identified dominant hospital-associated pathogens (HAPs) and
80 summarized their putative routes of transmissions. Those sources mainly
81 include physicians' and staff's clothing [11–13], stethoscopes [14], phones [15–
82 18], keyboards [19] and even patient bedrails [8]. In any case, the solid
83 surfaces accumulate a large spectrum of microbiota, which naturally favors
84 horizontal gene transfer (HGT) via mobile genetic elements (MGEs). Patients
85 can acquire ARGs by "direct contact", but those "invisible aerosols" also have

86 the chances entering into patients. To our best knowledge, the detailed
87 temporal aerosol ARG profiling variations with genus and/or species level
88 information in hospital environment seem to be lacking currently.

89 Traditional studies in researching ARGs, HAIs and HAPs have relied heavily
90 on cultural isolates and molecular gene identifications [20–25]. However, the
91 'needle in a haystack' cultural, PCR and quantitative PCR (qPCR) screening
92 methods demand intensive labor and are not cost friendly. Even though high
93 magnitude qPCR technology and microarray can somehow relieve the low
94 throughput issue, the lack of assay controls and arbitrary detection threshold
95 setting are relatively invalid to resolve inaccurate and false positive signals
96 [26,27]. Metagenomics sequencing focuses more on the qualitative patterns
97 and the signals are also comparable given same sample handling and
98 operational protocols. Also, metagenomics sequencing can generate sufficient
99 information regarding entire community and gene functionality, helping us
100 understand the potential pollution sources and distribution patterns thoroughly.
101 In this study, we mainly surveyed one-year aerosol samples from three
102 different departments in a large-scale municipal hospital. Besides aerosols, we
103 also collected water and hospital surface samples. We applied cultivation,
104 PCR, qPCR, Nanopore sequencing and Illumina metagenomics approaches to:
105 1) identify and compare ARGs and associated carriers in different departments;

2) study the temporal and spatial microbial community and functionality variations; 3) emphasize aerosol ARG distribution patterns within hospital.

Results and discussion

The prevalence of antimicrobial organisms and evaluations of applied methodologies

We have cultivated total of 102 (12 are blank controls) antibiotic selective LB plates targeting 15 samples (aerosol, water and surface samples) from 6 departments. All negative controls (no antibiotic added) showed colony and/or plaque morphology. From Table S1, we can see tetracycline and erythromycin had highest detection rate in all samples (both 40%), and other antibiotic detections were not far behind. The antimicrobial organisms were all bacterial colonies in water samples of the Outpatient hall (M_WO) and door handles seemed to mostly have tetracycline resistance in eukaryotic cells. Notably, the winter and summer aerosol samples in Pediatrics department (P_A) showed completely different ARG carriers (from bacteria to eukaryotes). Antibiotic resistances were commonly detected using cultural method and some of the samples have resistances from both bacterial and eukaryotic cells (e.g., O_A1 and P_A2). Equal number of bacterial colonies and eukaryotic plaques (20%) were found from all samples (blank control excluded). We also amplified total

of 21 samples, including aerosol, swabs and water samples by full length rRNA PCR. 11 out of 21 (52.4%) were bacterial positives, and eukaryotes showed a higher detection rate (71.4%). Culture negatives of M_A1 and M_A2 showed signals using PCR. Besides M_A1 and M_A2, the swabs of P_D and CAN_D samples also showed similar results, but most of the samples demonstrated solid consistency with cultural isolations. In this study, cultivation and PCR methods identified both bacterial and eukaryotic resistances from aerosol, with comparable detection rates to water and surface samples, suggesting aerosol might be a potential transition route in hospital setting, which was also consistent with others [8,20,21,50–54]. The same 20% detections for both bacterial and eukaryotic resistances suggest resistant eukaryotes might be an overlook. PCR techniques showed higher detection rates (at least 12% higher), indicating PCR has better methodology sensitivity but apparently, PCR is not able to distinguish live or dead cells.

For qPCR assays, we amplified 15 samples (Table S2), but only obtained positive results from *intl1, tetW* and 16S assays. One of the explanations might be we have only applied the correct candidate primers regarding those two assays since both *intl1* and *tetW* primers used here were widely applied in hospital settings (other primers were designed for non-hospital environment). Therefore, our results indicated the selection of specific hospital ARG primers

145 was necessary since ARG genotypes varied greatly [29]. All standard curves
146 (*intI1, tetW* and 16S) have higher R^2 values (> 0.983), but the amplification
147 efficiencies varied from 1.1 to 2.0. The lowest limit of detection (LOD) value is
148 10^4 gene copies/reaction. 13 out of 15 samples were *intI1* positives (two water
149 samples not amplified), and aerosol samples showed higher concentrations
150 compared to other types (data not shown). Similar detection trends were found
151 with *tetW*, but we noticed two water samples were positive this time (100%
152 detection). ARG qPCR results showed winter samples possessed relatively
153 higher concentration regarding all sample types, and aerosol samples can be
154 quantified better than water samples. One of the possible reasons could be the
155 aerosol microbes were accumulated longer on solid surfaces than water
156 samples. However, *tetW* was able to be identified even in water samples,
157 suggesting tetracycline was very prevalence and dominant in this hospital. We
158 also applied metagenomics approach to profiling ARGs, and the main present
159 ARG subtypes were multidrug (all year long) and clear temporal aerosol
160 distribution patterns within different departments were noticed [55]. In reality, it
161 is impossible to cultivate all antibiotic samples, but cultivation is the best way to
162 illustrate the correct antibiotic phenotypes. In our study, metagenomics
163 sequencing only generated short reads and required computational resources
164 to reconstruct the “broken” genomic fragments. Therefore, inconsistencies

between cultural and metagenomics sequencing are unavoidable, which is supported by a newly published work [56]. The varied sensitivity and specificity values from cultural, PCR and sequencing methods suggest using only one of the techniques may cause inconsistent interpretations. Therefore, we also applied Nanopore sequencing to confirming the ARGs that were carried by either bacterial genomes or plasmids. Using Nanopore sequencing, we successfully assembled 5 bacterial genomes and detected 25 potential plasmid-like fragments, confirming the prevalence of ARGs and necessity of applying multiple methodologies. We applied very strict rules searching those plasmid-like fragments in order to generate confident interpretations. We also aimed to support that sample handling and DNA extraction were able to play important roles in interpreting results. For instance, some environmental research groups recommended limited freeze-thaw cycles and relatively mild cell lysis steps [57]. In this study, *tetW* qPCR had good correlation with metagenomic sequencing, indicating metagenomics sequencing has the ability to accurately detect gene concentrations quantitatively. However, since tetracycline resistance was such dominant and concentrated in this hospital, it seems sequencing may have quantitative ability only if DNA quantity and quality were guaranteed. Notably, we did not identify the *tetW* carrier, and the one of the possible reasons could be carrier fragments were “broken apart”

during sample handling. Overall, using all widely applied methods together, we proved ARGs were prevalent in almost every possible media (air, water and surfaces) within hospital setting, and the winter aerosols had the most diverse and concentrated ARGs in all tested departments.

Aerosolized antibiotic resistance threats in this hospital

From metagenomics analyses, we saw winter aerosol samples had much higher ARG diversity and abundance than summer samples overall. For instance, among 1208 total ARG subtypes in database, P1 had 167 hits, followed by 144 detections in M1 and only 42 in O1, but P2 had 384 hits followed by O2 and M2 with 357 and 344, respectively. 727 out of 1208 subtypes were not present in all aerosol samples, and only 36 detections were found in all samples, suggesting a highly diversified aerosol resistome in hospital setting. The common identified included 1 bacitracin, 2 aminoglycoside, 2 chloramphenicol, 2 fosmidomycin, 1 MLS, 1 sulfonamide, 1 unclassified and 26 multidrug subtypes. If grouped by sampling seasons, 86 occurred in winter (besides mentioned subtypes above, quinolone, tetracenomycin, tetracycline, trimethoprim and vancomycin subtypes were newly found) and only 11 in summer (only β-lactam and multidrug subtypes). Notably, qPCR results showed a similar trend, and a R^2 value of 0.72 was obtained between normalized qPCR (*tetW* per 16S rRNA gene copy) and *tetW*

abundances determined by ARG-OAP (tetW per 16S rRNA gene copy). Even though ANOSIM showed no significant differences among all aerosols (p = 0.67), the ordination from PCoA analysis was still able to visually separate seasonal variations. In general, each department has relative unique resistome especially in summer. As shown in Figure 1A, winter aerosol samples clustered closely in regard to both aerosol ARG genotypes and community (OTU-based), while summer samples from different departments showed evident scattering. Additionally, hospital aerosol resistome is distinct from other environments but closely relates to human-related samples (Figure 1B), highlighting the direct impact of patients visiting on hospital aerosol resistome. From Table 1, the commonly present ARG carriers identified in this study included genera of *Escherichia*, *Bacteroides*, *Staphylococcus*, *Corynebacterium*, *Micrococcus* and *Acinetobacter*, which were also clearly noticed in other studies [20,54,55]. Corresponding to its high patients visiting and personnel exchange frequency, Outpatient hall (main entrance of the hospital) holds the most diverse and concentrated carriers, emphasizing the necessarity to reinforce resistome management under high visiting frequency [51]. Pediatrics and Ophthalmology showed more or less similar community composition (Figure 1A and Figure S2) than Outpatient hall, which was also confirmed by ARG source tracking results (Figure 2). Their differences with

225 Outpatient hall especially in summer suggest the applied treatments in
226 different departments could also change aerosol bacterial communities (also
227 confirmed by functionality profiling) and resistome.

228 Top 50 MetaPhlAn2 microbial community results (species level, totaling 1235
229 species) can be seen in Figure S2. Most of the species on list are bacteria, but
230 we also detected polyomavirus and porcine type-C oncovirus at relatively
231 higher abundances. In the list, genera such as *Staphylococcus*, *Streptococcus*,
232 *Acinetobacter* and *Pseudomonas* were also commonly present in hospitals.
233 For instance, some of the species were from known sources, such as mouth
234 (*Streptococcus sanguinis* and *Rothia dentocariosa*) [58,59], skin
235 (*Acinetobacter lwoffii* and *Propionibacterium acnes*) [60,61]，clinical/hospital
236 setting (*Acinetobacter johnsonii*, *Pseudomonas stutzeri* and *Acinetobacter junii*)
237 [60,62] and air dust (*Aerococcus viridans*, *Enhydrobacter aerosaccus* and
238 *Micrococcus luteus*) [63]. From HUMAnN2 results, a clear trend of aerobic
239 lifestyle was observed in aerosol community of the main entrance at both
240 summer and winter with aerobic respiration, TCA cycle and biosynthesis (GDP
241 mannose, L-valine, fatty acid etc.) were the dominant metabolism pathways. It
242 is interesting to observe some prevalent anaerobic pathways in the aerosol of
243 summer Pediatrics department such as Sulfate reduction, homolactic
244 fermentation and pyrimidine deoxyribonucleotide, implying a non-negligible

245 microbial accumulation in nutrient-deficit air dust in summer hospital setting.
246 But this anaerobic metabolic trend disappeared in wintertime, and this
247 probably was caused by temperature decrease. Such growing effect could
248 also be observed by α diversity analysis (see SI), which the summer samples
249 tended to have simplified community with sharply reduced Shannon index than
250 winter samples (Table S3). It seems the HAIs and HAPs are closely associated
251 with visitor numbers [8,51], nonetheless, we are facing severe aerosol
252 antibiotic resistance threats, especially in winter. Our results demonstrated we
253 might need to apply different strategies to address ARG contamination
254 spatially and temporally within hospital setting, especially more effort should
255 be made towards Outpatient hall.

256 Cultivation method was able to screen antibiotic resistances; PCR and qPCR
257 could provide ARG and categorical microorganism information; sequencing
258 analysis helped us with a variety of community composition and functionality
259 knowledge. However, we still need to bridge which specific microorganisms to
260 carry which specific ARGs. In our study, we had very strict rules in determining
261 potential carrier by metagenomics approach. For instance, in our novel
262 genotype sharing network analysis, we incorporated MetaPhlAn2 to re-ensure
263 the correct carriers, and we have noticed strong consistency between two
264 state-of-art sequencing analysis methods (Figure 3, Figure S2 & S4),

265 illustrating quality and valuable datasets were obtained in this study. Even we
266 feel confident about bacterial carriers; we are still missing eukaryotic antibiotic
267 resistance carriers. One of the possible explanations could be eukaryotic cells
268 were not studied thoroughly in current stage. The potential carriers identified
269 by rRNA gene based analysis were comparable with other work [50,64], but as
270 shown in α diversity results we only obtained class level confidence (Table S3).
271 Therefore, high resolution of eukaryotic composition databases were needed,
272 as West et al. [65] have already stepped up and more eukaryotic
273 metagenomics sequencing related effort should be made in future.

274 **ARG temporal and spatial distribution patterns**

275 In this study, we performed three types of source tracking analyses based on
276 OTU, ARG and pathogen abundances, respectively (SI File 1 & 2). As
277 introduced, we treated two season M aerosol samples as “source” and other
278 departments as “sink” for SourceTracker. “Unknown” is a default output,
279 representing potential sources besides initial targets. From community source
280 tracking perspective (Figure 2 OTU), although Pediatrics winter aerosol (P2)
281 still detained summer sources, the overall impact of Outpatient hall source
282 increased greatly from summer (around 20%) to winter (around 50%); similar
283 trend was also observed in source tracking based on aerosol resistome
284 (Figure 2 ARG). M source explained more than 80% winter resistome from

285 Pediatrics and Ophthalmology departments while it was roughly 45% in
286 summer. Such huge impact on overall aerosol community and resistome from
287 Outpatient hall/Main entrance of hospital at winter indicated a relatively
288 “transient” feature of aerosol microbiome, which was largely introduced by
289 patients visiting via the Outpatient hall [66,67]. This observation was in
290 accordance with the closely clustered winter resistome revealed by PCoA
291 analysis (Figure 1A). To the contrary, the summer aerosol community is more
292 “resistant” to the influence from the entrance source which probably owned to
293 the microbial accumulation revealed by previous community functional
294 analysis and in accordance with the scattered resistome by PCoA results
295 (Figure 1A). Also, in a review paper from Lax et al. [7], the authors claimed
296 opening the windows in hospital indoor rooms has been found to significantly
297 link to the reduction of the percentage of pathogenic airborne bacteria. In this
298 study, the air conditioning systems at Shenzhen city operate over half of the
299 year at summer, indicating the closed window condition happens most of the
300 summer. Therefore, we have noticed summer pathogen persistence in
301 Ophthalmology department, which is also observed by Gao et al [55].
302 Additionally, a distinct pattern was noticed from pathogen source tracking that
303 unknown sources were dominant except for E2 which showed strong evidence
304 that the summer pathogen still persistent from summer to winter underpinning

305 the notion that persistent pathogens could dominant in hospital setting from
306 days to months [68]. Those “unknown” could be sourced from “direct contact”
307 happened within hospital as prior studies suggested [8,64]. Additionally, some
308 new opportunistic pathogens may also challenge the SourceTracker algorithm
309 because we only focused on those well-known targets at the moment. In
310 summary, ARG and community source tracking results illustrated Outpatient
311 hall could serve as a major pollution source all year long. Besides source
312 tracking, we also applied Procrustes analysis to illustrate temporal and
313 departmental variations when compared community to resistome. Even though
314 significant association still exists ($p < 0.01$) between aerosol community and
315 resistome, Procrustes analysis suggested the summer samples showed a
316 much weakened association than winter samples as indicated by a larger
317 measure of fitness by M^2 value (the sum of square distance between matched
318 sample pairs in Procrustes transformation). The findings implied a higher level
319 of inter-community ARGs exchange in summer hospital aerosol. Such
320 increased ARG-HGT level probably caused by microbial accumulation in
321 summer, which aerosol did not associate with increased resistome as the
322 winter aerosol showed much higher resistome abundance and diversity than
323 summer samples.

324 To illustrate the ARG genotype exchange pattern, we constructed ARG

genotype sharing network with over 99% similarity to a known genotype in SARG database to map the interconnection among departments based on metagenome-assemblies (Figure 3 and Figure S4). Outpatient hall had largest number of ARG genotypes and the network was the most complicated. Our strict algorithms detected commonly present genera such as *Staphylococcus*, *Micrococcus*, *Streptococcus* and *Enterococcus* in hospital setting. *Staphylococcus* carried the largest number of ARGs (14 ARG genotypes) compared to other carriers. Fewer genera were identified in Ophthalmology and Pediatrics department (Figure 3), which corresponded to the results in Table 1. ARGs resistant against Aminoglycoside, bacitracin, β-lactam, chloramphenicol, MLS, multidrug, tetracycline, bleomycin and sulfonamide were commonly detected in this study (Table 1 and Figure S4). Consistent with other main techniques used in this study, winter aerosols had higher concentrations overall. High abundance of *Micrococcus* positively correlated with high ARG genotype concentrations (Table 1). Notably, most of the ARG genotypes were found from both summer and winter samples. In contrast to *Tet40, erm(36) and lnuB* which were only carried by single microorganism, some instances of Inter-species HGT could be confirmed in hospital aerosol samples, such as, *tetK* HGT between *Staphylococcus*, which was further confirmed by whole genome Nanopore sequencing (Figure S5). The most

345 commonly shared ARG genotypes among different departments in hospital
346 aerosol mainly included *aadD, CE, tetK, tetA, tetZ and norA.* Since plasmid
347 mediated *CE* transmission was observed between *Staphylococcus* and
348 *Corynebacterrium* within Outpatient hall in this study, we infer plasmids carried
349 CE and tetracycline resistance could possibly lead to ARG transmission
350 among departments. Meanwhile, we have successfully assembled 5 bacterial
351 genomes with *Enterococcus faecalis* and *Staphylococcus saprophyticus* being
352 assembled into complete circular format. Also, the antibiotic phenotypes all
353 corresponded to the genotypes that were identified from either genomes or
354 plasmids. As shown in Table S6, tetracycline, aminoglycoside, macrolide and
355 β-lactam were all detected from these live cells, and tetracycline resistance
356 genes were frequently found. Most of the inter-species and inter-departmental
357 ARG sharing was mediated by plasmids that were previously identified to
358 associate with *Staphylococcus saprophyticus,* suggesting the movement of
359 *Staphylococcus saprophyticus* plasmid among departments may be an
360 important HAI transfer pattern and causing infections in this hospital. Genera
361 such as *Staphylococcus*, *Xanthomonas* and *Enterococcus* were also
362 endangering hospital environment (confirmed by both Illumina and Nanopore
363 sequencing). Nanopore sequencing results also supported *tet(K)* was shared
364 among hospital departments via the presence of *Enterococcus* and

365 *Staphylococcus.* These results suggested despite the high-throughput
366 coverage offered by metagnomic sequencing, we still need to apply culture
367 isolate sequencing to confirm the existence of plasmids. Using much longer
368 read length Nanopore sequencing, we have found similar plasmid transferring
369 phenomenon among departments and assured the existence of plasmids
370 (Figure S5 & S6). In combination of two sequencing technologies, our
371 genotype sharing network confirmed the plasmid-mediated transferring
372 mechanism in hospital air for the first time. Wealth prior studies mostly either
373 applied amplicon sequencing technologies to identify aerosolized microbial
374 communities [8,54,64]; or used PCR based methods to profile resistome
375 [51,69]. Also, there are some studies applied metagenomics approaches
376 [50,70] on aerosolized microbial community in hospital, but they did not
377 connect ARGs with specific carriers or utilized correlation network analysis to
378 connect ARG carriers to bacteria [55,71]. Based on these frontier works, in
379 this study we integrated state-of-art metagenomics to localize ARGs carried by
380 specific bacteria using strict algorithm and further confirmed the ARG
381 transferring mechanism using culture-based whole genome Nanopore
382 sequencing. The microbial community and gene functionality results from
383 aerosol metagenomics results identified an obvious changing trend from
384 summer to winter. Our effort will encourage more metagenomics applications,

385 but researchers need to be more cautious interpreting results and selecting
386 correct inputs.

387 **Best management practice to alleviate hospital ARG issues**

388 All results clearly demonstrate the antibiotic resistances dissemination via
389 aerosol was a concern in hospital. Some groups also modeled the
390 microorganism concentrations with other related factors, such as human
391 occupancy, visiting patients among departments, indoor temperature etc.
392 [8,51], but the results only showed weak correlations. Since our study site is
393 highly compacted and intensively used every day, strict control on visitors flow
394 may not be applicable in most public hospitals. Therefore, constraining the
395 ARGs spread within certain area seems to be important. Some practices, such
396 as establishing anteroom inside of patients' room seem to work greatly [53].
397 Researchers also recommended providing positive air pressure to reduce
398 pathogenic transmissions [7,53] as they found humidity and outdoor air
399 fractions had strong temporal patterns and strong correlations between rooms
400 [7], which was also confirmed in this study. Besides, hospital indoor water sink
401 is another potential aerosol ARG source as discovered by Kotay et al. [72].
402 The splashed droplets could lead to the transmission from contaminated hand
403 washing sinks, which was also supported by our PCR based resistome
404 detection method. Additionally, determining cleaning strategies according to

405 specific microbial resistome carriers might be important, however, unlike
406 bacterial resistome, we found current metagenomic sequencing approach
407 cannot identify the potential Eukaryotic carriers even though studies have
408 already proved some resistance like kanamycin can be carried by eukaryotic
409 cells and can be transferred between prokaryotes and eukaryotes [73,74].
410 Therefore, such lack of advanced eukaryotic carrier knowledge might be a
411 factor that hinders the application of efficient cleaning strategies.

412 **Limitations and future directions**

413 Even our culture-facilitated comparative metagenomics method seemed to
414 work well in resistome patterns, Illumina sequencing still needs more
415 turnaround time (lab work and analysis). However, in hospital related
416 applications the longer procedure might cause issues, especially for quick
417 disease identifications and evaluations. The short turnaround time and much
418 longer sequence read favors studying the structure and genomic context of
419 resistance determinants, which was not able to be addressed by Illumina
420 sequencing. Because antibiotic regions are often flanked by repetitive insertion
421 sequences and cannot be resolved by standard short read methods [75,76].
422 Therefore, we recommended developing comparative metagenomics based
423 on the quick Nanopore sequencing to perform hospital applications in future.
424 Meanwhile, a yearlong temporal and spatial micro-ecology trend in a newly

opened hospital suggested there was basically no stable universal bacteria transmission pattern within hospital [8]. Even though our results correlated to other aerosolized findings in China (Table S4), we still speculated that our one year survey only demonstrated limited aerosol ARG patterns, meaning multiple survey years and more sampling frequencies were needed in future in order to identify a stable pattern in large-size hospitals.

Conclusion

The overwhelmingly present ARGs and bacterial carriers in hospital clearly suggest an urgent threaten to human health. The seasonal ARG and micro-ecology changes identified in our study delineated a potential plasmid mediated ARG transferring among departments temporally and spatially. Traditional detection methods, e.g. cultural and PCR related technologies etc. justified the metagenomics approaches, and the state-of-art analyzing methods with strict cutoffs are able to assisting metagenomics approaches to providing more accurate interpretations.

Methods

Site description, sample collection and handling

Two sampling rounds were performed on summer (Aug. 2018) and winter (Jan. 2019) at Shenzhen Hospital of Peking University, respectively (only two

444 distinct seasons based on ambient temperature at Shenzhen city). The
445 hospital was operated at the end of 1999 and serves over 8000 persons daily.
446 It has around 50 functional departments, and the total visitors reached 2.92
447 million in 2018. Three types of samples were surveyed in this study, including:
448 1) inlet and outlet waters from fountains in Outpatient hall/Main entrance (abbr.
449 as M), Ophthalmology (abbr. as O) and Pediatrics (abbr. as P) departments; 2)
450 door handle and lobby chair surface swabs collected using sterilized cotton
451 from M, O, P, Cancer center, Male reproductive center and Inpatient
452 departments (six departments); 3) air conditioner filters from M, O, and P
453 departments in two distinct seasons (1 denotes summer and 2 denotes winter
454 sampling) with inpatient department filter sampled only in winter. In this
455 hospital, Outpatient hall (M) is the main entrance to all facilities/departments,
456 and Pediatrics department has more patients compared to Ophthalmology
457 department, which serves around 4000 inpatients yearly. Water samples were
458 collected in sterilized container, and cotton swabs were sampled as previously
459 described [1, 2]. As the main study targets, air conditioner filters (see “filter dirt”
460 in Figure S1) were firstly detached by cleaning expert and then transported
461 back to our lab with swabs and waters on ice within 2 hours after sampling.
462 After received at lab, the dirt on filters were shaken and washed off into
463 sterilized autoclavable containers using sterilized ddH2O. Thereafter, the

nonsoluble and dissolved dirt samples were filtrated immediately using 0.22 μm filters (Millipore, USA). Cotton swabs and the filtrated membranes were then transferred to purple-capped tubes in FastDNA kit (MP Biomedicals, USA) and stored at -20 °C before downstream DNA extractions (see Figure S1 for overall schematic overview).

Metagenomics sequencing data analyses

For cultivation, DNA extraction, PCR and sequencing lab work, please refer to supporting information (SI). We used Illumina HiseqXten-PE150 platform to perform aerosol metagenomics sequencing (Novogene, Nanjing, China). The sequencing library size was 350 bp and 10 G raw data was initially aimed (see Table S7). Each department was designated to have two samples. We also performed two replicates in M and P on winter sampling to testify the reproducibility of our methodology, and one randomly picked replicate from M and P winter samples was used for subsequent analysis. In this study, metagenomics analyses were based on reads and metagenome-assemblies, respectively. All reads were QC qualified before application [28]. The following four subsections focused on read level analyses, and the last subsection dealt with assembly analyses.

ARG detection

The rapid characterization and quantification of ARGs were analyzed by ARGs-OAP v2.0, which was developed by Yin et al [29]. Briefly, both 16S and ARG type/subtype information were extracted from metagenomics reads at first. And then, the normalized ARG profiling was calculated by normalizing the extracted ARG-sequences against reads mapped to 16S rRNA gene (V6 region). Both ARG type and subtype (equivalent to genotype) results were summarized in SI File 1.

Community structure and functionality analysis

MetaPhlAn2 was used to compute the composition of microbial communities with species-level resolution [30]. Since MetaPhlAn2 can generate species-level resolution, we searched the pathogen species in the microbial community profile to generate an aerosol pathogen table with abundance information (SI File 2). The community profiling results can be visualized in Figure S2. We also applied HUMAnN2 to profile microbial pathways using UniRef90 and ChocoPhlAn databases with the default protocols [31].

rRNA identifications and OTU analysis

In this study, we used SortMeRNA (version 2.1b) [32] to extract both 16S and 18S rRNA sequences from metagenomics reads. silva-bac-16s-id90.fasta and silva-euk-18s-id95.fasta databases were used with --fastx --paired_in --aligned

output --log -a 8 parameter settings. Extracted 16S and 18S sequences were then analyzed by vsearch (version 2.8.0) [33] to generate OTU tables for all tested samples (identity cutoff=0.97). OTU were then analyzed by alpha_diversity.py program in QIIME (1.9.1) [34] to calculate Good's coverage and Shannon index values (α diversity).

Bayesian source tracking

In this study, we focused on three types of source tracking analyses using SourceTracker [35]. Since patients, doctors and visitors all start their routines at Outpatient hall, we assume it can serve as the "source" in Bayesian algorithm (both cultivation and PCR results confirmed our assumption), leaving other departments as "sink" in algorithm. Each vsearch generated OTU table was firstly sorted based on abundances, and then commonly shared information was extracted and reorganized into matrix format for all samples (SI File 2). Besides community source tracking, we also took ARG genotype profiling from ARGs-OAP v2.0 and pathogen detection tables into source tracking analyses. SourceTracker R code (https://github.com/danknights/sourcetracker) was used to generate the bar chart (Figure 2).

ARG carrier and plasmid identifications from metagenome-assemblies

We assembled metagenomics sequencing reads separately (clustered by departments). Since we have replicates in winter sampling, we assembled two winter replicates with one summer sample together regarding M and P. All assembly processes were performed using CLC Genomics Workbench 12 (Qiagen Bioinformatics, USA). The program was run by default setting with minor changes: 1) the minimum contig length was set to 500 bp instead of 200 bp; 2) length fraction (mapping process) was set to 0.8. After we obtained M, O and P aerosol metagenome-assemblies using de novo assembly method, open read frames (ORFs) in each assembly were predicted by MetaGeneMark [36]. Diamond [37] was then applied to search these ORFs against RefSeq protein (July, 2017) database (E=10-5). The blast format output of Diamond was parsed by MEGAN5 [38] for taxonomic assignment. Then assembled contigs were taxonomically classified to a Kingdom if more than 50% of the genes within a contig were attributed to the same Kingdom. The same threshold were used for Phylum and Class level taxonomies, while 0.4 was applied for order, followed by 0.34 for family, 0.3 for genus and 0.25 for species [39]. Taxonomic annotations generated from this homology-based method were served to identify phylogenetic affiliation of ARG carrying bacteria. BlastP [40] was used to search ORFs against SARG database with similarity cutoff of 80% and alignment cutoff of 70% to identify ARGs from these

541 metagenome-assemblies. A much stricter similarity cutoff of > 99% was used
542 when constructing the ARG genotype sharing network. Genus level carrier with
543 two seasonal sequencing read partition results (normalized after mapping
544 reads to contigs) were summarized in Table 1 [41]. In this study, PlasFlow.py
545 program was used to predict the existence of plasmids in all
546 metagenome-assemblies [42]. In order to increase result accuracy, we
547 changed --threshold probability parameter to 0.9 (0.7 by default [43]). The
548 assemblies were then subdivided into chromosomal and plasmid contigs, and
549 the plasmid contigs were used for further genotype sharing network analyses.

550 **ARG and plasmid identifications from isolates**

551 From cultivated LB agar plates, we randomly selected 11 isolates (antibiotics
552 added or free) for Nanopore sequencing. Briefly, the selected single bacterial
553 colony was firstly enriched in LB broth and after cell precipitation by centrifuge,
554 whole genome DNA extraction was performed as mentioned in previous
555 section. Each bacterial species was determined by Sanger sequencing
556 (targeting full length 16S rRNA fragment) before Nanopore sequencing. The
557 QC passed Nanopore sequencing fast5 files were basecalled with albacore
558 [44]. Next, porechop (https://github.com/rrwick/Porechop) was used to trim
559 adapters. The resulted fasta were assembled by Canu 1.8 [45]. Since we
560 performed Sanger sequencing, the estimated -genomeSize option in Canu

561 was correctly imported for correction, trim and assemble steps. A recent
562 published plasmid database - PLSDB [46] was used to search plasmid-like
563 DNA fragments (>90% similarity and 6000 bp in length for successfully
564 assembled contigs or >2000 bp for Nanopore raw reads). We then incorporate
565 the ARGs carried by plasmid-like DNA results into further analyses. Since
566 gene calling might not be reliable for Nanopore dataset, ResFinder 2.1 [47]
567 was used to identify ARGs from bacterial genomes/Nanopore reads (90%
568 similarity and 60% alignment cutoff). The detail results were summarized in
569 Table S5 & S6.

570 **Statistics and network analyses**

571 In order to confirm the reproducibility between replicates, Spearman's
572 correlation tests were performed using cor function in base-R (3.5). We
573 checked the community and ARG correlations, respectively on all replicates
574 (Figure S3). Meanwhile, to compare custom aerosol communities in different
575 sampling sites, we performed analysis of similarities (ANOSIM, R package
576 "vegan" [48]) using shared bacterial OTU matrix. For consistency, same matrix
577 was also used for source tracking. Procrustes analysis was performed (using
578 procrustes function in "vegan" package) to illustrating correlation between
579 temporal aerosol resistome and community profiling [49]. In brief, the
580 Procrustes transformation significance was calculated by measuring M2

between principle coordination analysis (PCoA) plots (both based on Bray-Curties distance) to a set of empirically distributed M2 values determined from 999 computational permutations. In each permutation, the sum of squared distances between matched samples (M2) was used to compute a p value (compared to original distances).

Genome sharing network was constructed to check the dissemination pattern of hospital aerosol resistome. In brief, 1) the two sampling events were clustered by normalized outputs generated from MetaPhlAn2 and ARGs-OAP v2.0; 2) only ARG hits with similarity greater than 0.99 were considered as genotype sharing among above normalized datasets. We also incorporated the potential plasmid information (computationally identified by PlasFlow.py program) to clarify the possible ARG transfer among hospital departments via plasmids. Meanwhile, the plasmid-like fragments identified from Nanopore sequencing were also integrated into genotype sharing network to confirming plasmid movements. The community correlating with ARG type results can be seen in Figure S4, and genotype sharing network results (with bacterial genus level information) can be seen in Figure 3. Besides ARG genotype sharing network, we also applied similar protocol to network ARGs that were identified by Nanopore isolate sequencing (Figure S5). The network graph was illustrated using online diagram software called “draw.io”.

Declarations

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Competing Interests

The authors declare no conflict of interest.

Authors' contributions

XL performed all lab work, data analyses and wrote the manuscript. ZW performed cultural isolations, samplings and water filtrations; CD performed samplings and filtration experiment. MZ, BZ, CZ, LZ, ZZ and YY helped sampling and culture related lab work. XY designed the whole study, and XL & XY planed data analysis processes. All authors edited the manuscript and approved the final draft.

Availability of data and materials

Supporting information file has been deposited in Figshare (https://figshare.com/articles/Supporting_information/11437932). The Illumina metagenomics sequencing raw reads were deposited into EMBL-EBI (https://www.ebi.ac.uk/) under the following accession number ERS4202782-ERS4202789. Cultural isolate Nanopre sequencing reads were deposited under accessions from ERS4202826 to ERS4202836.

Ethics approval and consent to participate

The manuscript dose not report data collected from humans and animals.

Consent for publication

Not applicable.

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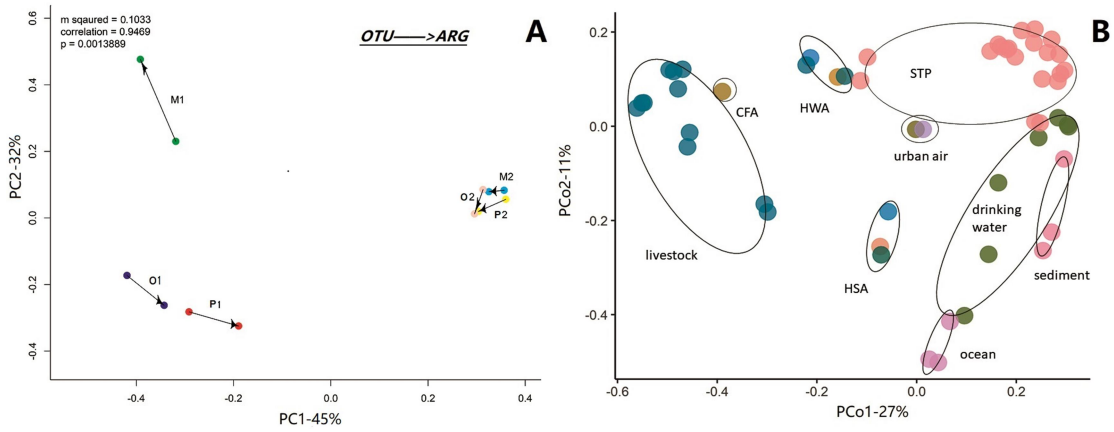
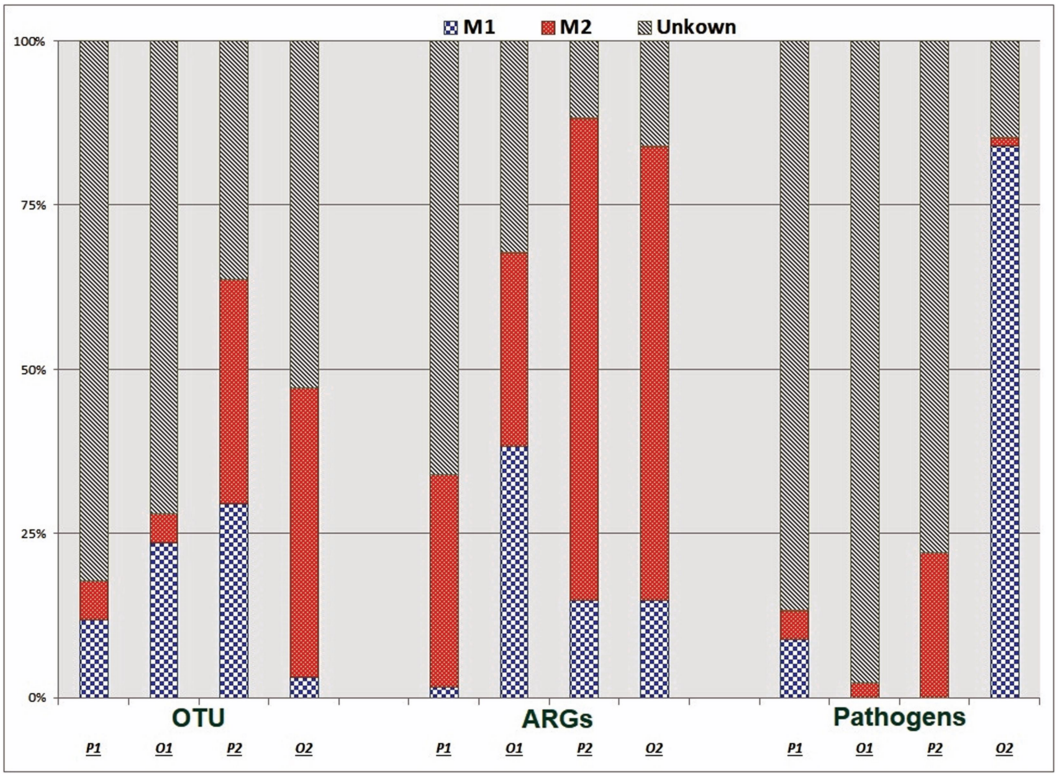
842 **FIGURE LEGEND**



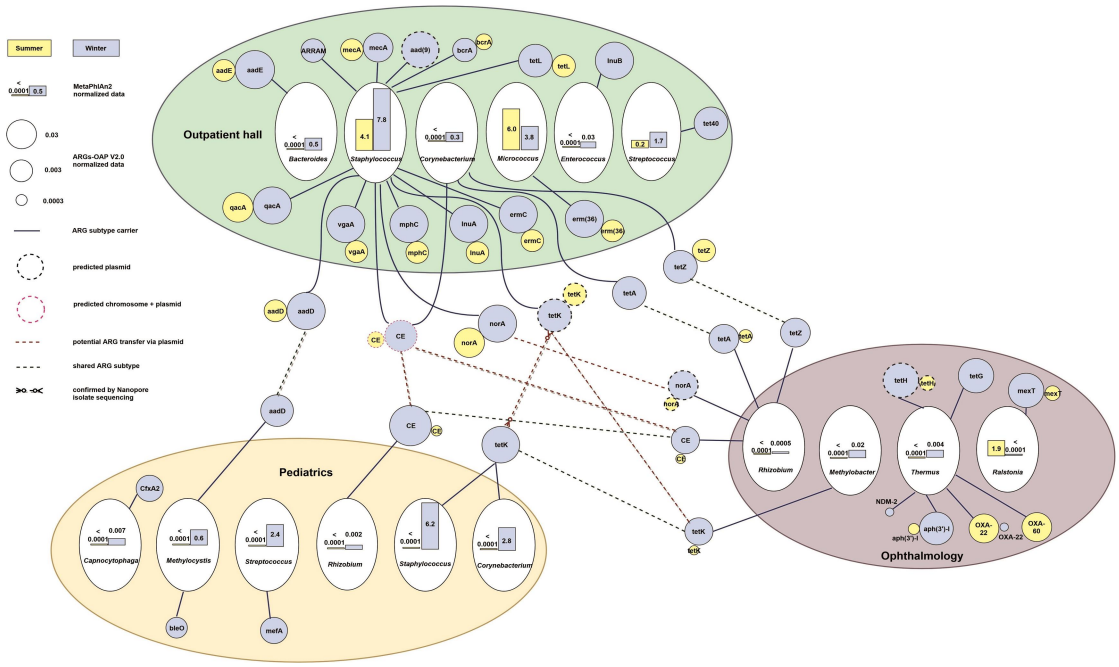
843

844 Figure 1 Temporal and spatial aerosolized comparative metagenomics results. Figure 1A is
845 Procrustes superimposition plot that depicts confident correlation between aerosol resistome
846 (Bray-Curtis) and community composition (Bray-Curtis); Figure 1B is principal coordinate
847 analysis (PCoA) based on ARG subtypes of various environmental samples: CFA represents
848 chicken farm air, HWA is hospital winter aerosols, HSA is hospital summer aerosols; STP
849 represents sewage treatment plant.



850

851 Figure 2 Temporal aerosol Bayesian SourceTracker algorithm results. M stands for Outpatient,
852 O represents Ophthalmology and Pediatrics is abbreviated as P. Two season's M aerosol
853 samples as source and other departments as sink for SourceTracker.



854

855 Figure 3 ARG genotype sharing network. Different colored oval represents different
856 departments studied. Vertical ellipse within each oval represents ARG genera that were
857 identified with their relative abundances in summer (yellow) and winter (blue), shown as bar
858 chart. Circle-pairs represent ARG genotypes. Genotypes are linked to this carrier as network
859 edges. Size of circle is proportional to its abundance (normalized against 16S rRNA gene)
860 within aerosol samples. Circle border represents the mobile mechanisms: plasmid carrying
861 (dotted line) and chromosome encoding (full line). ARG genotypes shared among departments
862 are shown in the middle area outside oval and each sharing instance was further linked with
863 dash lines; those plasmid (red) and chromosomal (green) carrying are shown as different
864 colors. Those sharing instances are confirmed by whole-genome Nanopore sequencing
865 (->o----o<-).

866 **TABLE**

867 Table 1 Aerosol ARGs and corresponding carriers identified by metagenome-asseblies in
868 different departments. count: contig numbers; contig per: ARG-carrying contig in percentage of
869 total contig; ppm%: the normalized part per million read percentage (see the corresponding
870 reference for more details).

ARG type	Outpatient hall						Ophthalmology						Pediatrics					
	genus	count	contig per, %	reads map to contigs, ppm%			genus	count	contig per, %	reads map to contigs, ppm%			genus	count	contig per, %	reads map to contigs, ppm%		
				M1	M2-1	M2-2				E1	E2					C1	C2-1	C2-2
aminoglycoside	*Escherichia*	93	1.95	0.76	1.05	0.07	*Xanthobacter*	268	**5.25**		7.18		*Methylocystis*	42	0.22		0.57	0.32
	Bacteroides	196	1.73	3.80	0.52	0.04	*Methylocystis*	85	0.76	0.06	2.03		*Francisella*	84	0.26		0.66	0.68
	Staphylococcus	939	3.68	0.15	1.67	1.50	*Pseudoalteromonas*	202	1.70		5.09		*Pseudoalteromonas*	30	0.32		0.74	0.43
	Acinetobacter	136	2.80	1.51	1.03	0.51	*Francisella*	14	0.53		1.39		*Thermus*	48	0.30	0.71	0.19	0.24
							Pseudomonas	162	**4.54**		**21.24**		*Moraxella*	32	0.31		0.56	0.86
							Moraxella	60	1.69		7.10		*Campylobacter*	289	0.45	1.61	1.16	0.88
													Xanthobacter	125	0.45		0.93	0.51
bacitracin	*Staphylococcus*	475	2.98	1.05	5.87	0.69												
beta-lactam	*Staphylo*	28	0.67	0.85	0.69	0.17	*Pseudo*	125	2.47	0.33	8.45		*Thermu*	76	0.26		0.36	0.59

	coccus						*monas*					*s*					
	Escherichia	74	0.55	1.33	0.06												
	Acinetobacter	122	2.13	3.81	1.98	0.05											
	Bacteroides	37	0.63	1.68	0.04												
bleomycin							*Methylocystis*	85	0.76	0.06	2.03	*Methylocystis*	42	0.22		0.57	0.32
chloramphenicol	*Corynebacterium*	29	0.48	0.55	0.67	0.09	*Rhizobium*	20	0.60		4.57	*Rhizobium*	100	0.25		0.32	0.55
	Staphylococcus	50	0.81	0.00	0.00	1.24	*Moraxella*	35	0.37		1.65	*Campylobacter*	289	0.45	1.61	1.16	0.88
	Lachnoclostridium	18	0.38	0.21	0.69	0.15	*Helicobacter*	20	0.71	1.78	0.25	*Helicobacter*	115	0.33		0.70	0.85
												Methylobacillus	238	0.60		0.35	0.55
												Moraxella	34	0.16		0.35	0.25
MLS	*Staphylococcus*	1194	**13.26**	5.30	1.53	0.31	*Treponema*	84	1.44		1.25	*Methylobacillus*	2365	**1.97**	14.98	0.33	0.53
	Micrococcus	169	0.89	0.09	2.38	0.11	*Flavobacterium*	85	**3.04**		8.28	*Xanthobacter*	84	0.53	0.47	0.81	1.01

	Enterococcus	2558	**11.02**	0.00	0.76	**10.21**	*Neisseria*	35	0.51		1.01	*Neisseria*	72	0.21		0.29	0.49
							Legionella	12	0.37		1.76	*Treponema*	164	**0.98**	**10.17**	0.13	0.27
							Acidiphilium	18	0.48	0.42	4.55	*Pseudoalteromonas*	18	0.17	0.49	0.43	0.18
							Xanthobacter	1641	**4.64**	0.04	**12.97**	*Aeromonas*	38	0.29		0.45	0.64
												Paracoccus	72	0.35	1.58	0.76	0.49
multidrug	*Staphylococcus*	1389	7.07	1.21	1.55	0.63	*Spirochaeta*	18	0.47		6.62	*Ralstonia*	307	**3.01**	0.14	1.91	1.78
	Acinetobacter	466	4.66	2.30	0.16	0.02	*Alcaligenes*	39	1.50	1.05		*Methylobacillus*	176	0.40		0.86	0.92
							Legionella	11	0.32		0.98	*Rhizobium*	21	0.19		0.27	0.46
							Pseudoalteromonas	11	0.33		1.24	*Spirochaeta*	61	0.70		0.89	1.17
												Fluoribacter	159	0.56		0.45	0.52
												Neisseri	112	0.56	0.09	1.44	0.83

												a					
												Methylobacterium	117	0.32		1.29	1.21
												Alcaligenes	60	0.56		0.24	0.74
												Methylomonas	130	0.28		0.47	0.54
sulfonamide	*Acinetobacter*	157	1.13	2.56	0.34							*Pseudomonas*	66	0.23		0.46	0.47
												Blastobacter	20	0.24		0.73	0.36
tetracycline	*Staphylococcus*	554	**10.41**	**12.17**	1.41		*Legionella*	31	0.94	0.14	**15.26**	*Legionella*	73	0.64		1.05	1.26
	Escherichia	28	0.52	0.20	2.38	0.18						*Methylobacter*	57	0.28		0.37	0.66
	Acinetobacter	2699	**12.19**	**23.86**	3.83	0.39						*Rhizobium*	149	0.80		0.48	0.57
	Corynebacterium	147	1.62	1.76	0.30	0.08						*Methylomonas*	42	0.44		1.03	0.97
	Streptococcus	33	0.43	0.79	0.35							*Methylobacillus*	454	**1.83**		3.70	3.05
trimethopri	*Escheric*	61	1.28	2.17	1.16	0.07						*Francis*	48	0.52		0.87	0.86

m	*hia*											*ella*				
unclassified	*Staphylococcus*	107	0.94	1.93	0.38							*Campylobacter*	39	0.41		0.79	0.62
	Escherichia	55	1.25		0.38	3.53											

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Figures

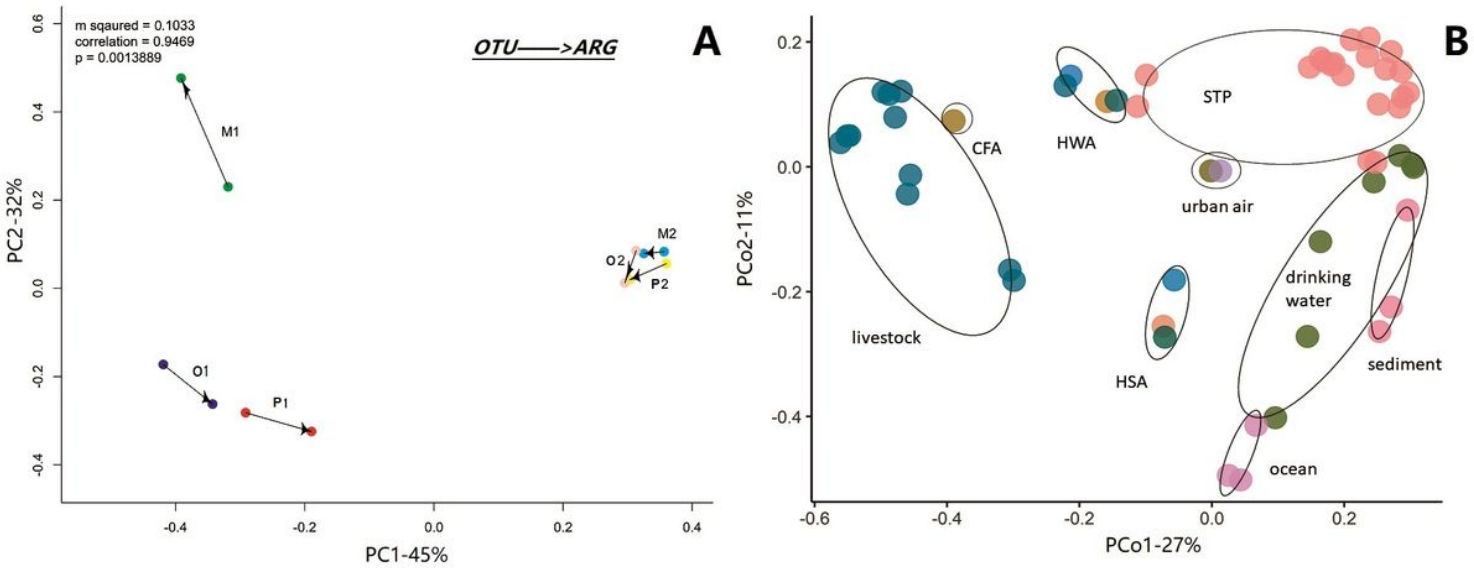

Figure 1

Temporal and spatial aerosolized comparative metagenomics results. Figure 1A is Procrustes superimposition plot that depicts confident correlation between aerosol resistome (Bray-Curtis) and community composition (Bray-Curtis); Figure 1B is principal coordinate analysis (PCoA) based on ARG subtypes of various environmental samples: CFA represents chicken farm air, HWA is hospital winter aerosols, HSA is hospital summer aerosols; STP represents sewage treatment plant.

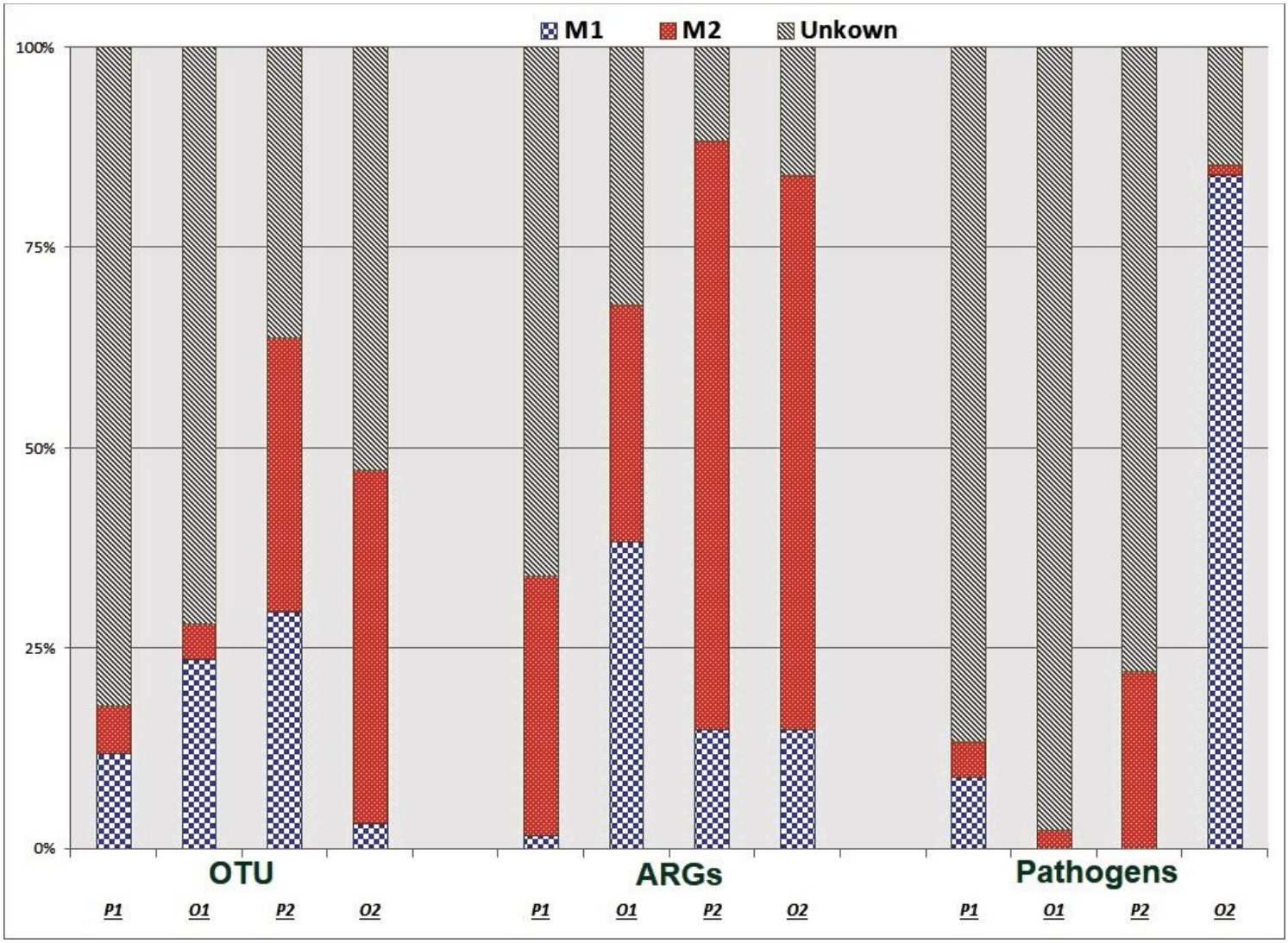

Figure 2

Temporal aerosol Bayesian SourceTracker algorithm results. M stands for Outpatient, O represents Ophthalmology and Pediatrics is abbreviated as P. Two season's M aerosol samples as source and other departments as sink for SourceTracker.

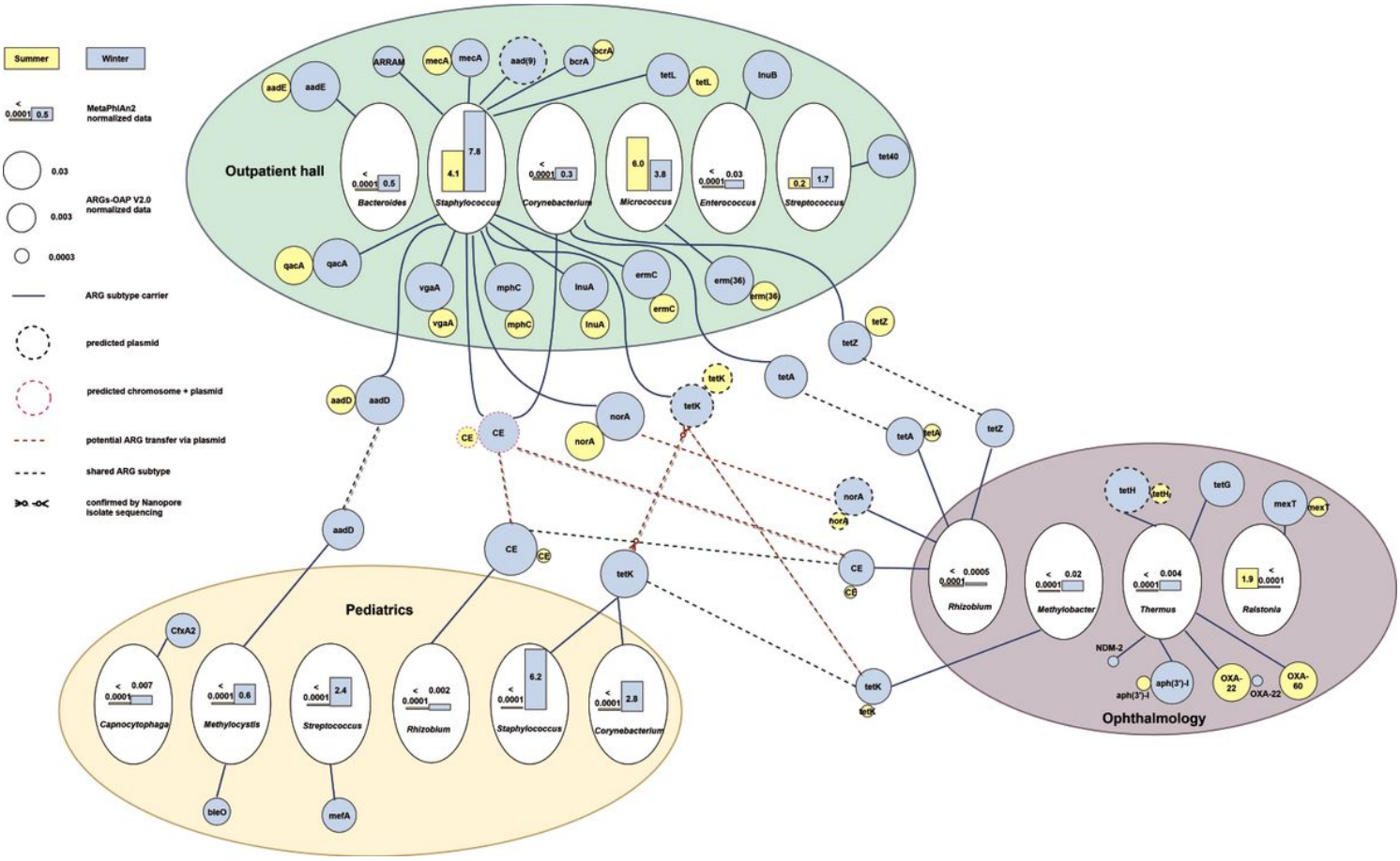

Figure 3

ARG genotype sharing network. Different colored oval represents different departments studied. Vertical ellipse within each oval represents ARG genera that were identified with their relative abundances in summer (yellow) and winter (blue), shown as bar chart. Circle-pairs represent ARG genotypes. Genotypes are linked to this carrier as network edges. Size of circle is proportional to its abundance (normalized against 16S rRNA gene) within aerosol samples. Circle border represents the mobile mechanisms: plasmid carrying (dotted line) and chromosome encoding (full line). ARG genotypes shared among departments are shown in the middle area outside oval and each sharing instance was further linked with dash lines; those plasmid (red) and chromosomal (green) carrying are shown as different colors. Those sharing instances are confirmed by whole-genome Nanopore sequencing (->o---o<-).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SIfile3HUMAnN2results.xlsx
- Table1.xlsx
- SIfile2pathogenandOTUlist.xlsx
- SIfile116SnormalizedARGsubtype.xlsx