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1 **Evaluation of ERIC-PCR and MALDI-TOF as typing tools for multidrug resistant *Klebsiella*
2 *pneumoniae* clinical isolates from a tertiary care center in India**

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10

11 **Abstract**

12 **Background and Aim:** Multidrug resistant *Klebsiella pneumoniae* is associated with nosocomial
13 infections in both outbreak and non-outbreak situations. The study intends to evaluate the potential of
14 enterobacterial repetitive intergenic consensus- polymerase chain reaction (ERIC-PCR), a genomic based
15 typing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)
16 proteomic-based typing techniques for clonal relatedness among multidrug resistant *Klebsiella pneumoniae*
17 isolates.

18 **Methodology:** Multidrug resistant clinical isolates of *Klebsiella pneumoniae* (*n* =137) were collected from
19 March 2019 to February 2020. Identification and protein-based phylogenetic analysis were performed by
20 MALDI-TOF MS. Genomic typing was done by ERIC-PCR and analyzed by an online data analysis service
21 (PyElph). Dice method with unweighted pair group method with arithmetic mean (UPGMA) program was
22 used to compare the ERIC profiles. The samples were also evaluated by PCR for the presence of genes

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23 encoding carbapenemases, extended spectrum beta lactamases (ESBLs) and mobile colistin resistance-1
24 (*mcr1*).
25 **Result and Conclusion:** Isolates were typed into 40 ERIC types, and six groups by MALDI-TOF-MS.
26 PCR-based analysis revealed that all the strains harbored two or more ESBL and carbapenemase genes.
27 None of the isolates revealed the presence of the plasmid mediated *mcr-1* gene for colistin resistance. The
28 study presents ERIC based typing as more robust in comparison to MALDI-TOF for finding the clonal
29 relatedness in epidemiological studies.

30 **Key Words:** Klebsiella pneumoniae, Multidrug resistance, ERIC, MALDI, Epidemiological typing

31 Introduction

32 *Klebsiella pneumoniae* is an opportunistic gram-negative bacterium accounting for approximately one third
33 of all hospital and community acquired infections. These include pneumonia, surgical wound infections,
34 meningitis, urinary tract infections and blood stream infections (1). Increase in the incidence and prevalence
35 of multidrug resistant (MDR) *K. pneumoniae* has been associated with therapeutic failures, prolonged
36 hospitalization, high mortality rates and a significant economic burden (2). In the last two decades, there
37 has been a tremendous increase in infections caused by multiple drug resistant gram-negative bacteria,
38 including those resistant to the last resort drugs like carbapenems and colistin (3,4). The World Health
39 Organization has prioritized *Klebsiella pneumoniae* as a target for the development of newer antimicrobials
40 for the treatment of nosocomial infections (5). Several transmission dynamics studies in both outbreak and
41 non-outbreak situations for ESBL-producing *K. pneumoniae* infections have shown the presence of high
42 clonal diversity (6–8).

43 Molecular typing techniques are powerful tools used to determine clustering among multidrug resistant
44 (MDR) *K. pneumonia*e and to derive information about their transmission. We used enterobacterial
45 repetitive intergenic consensus- polymerase chain reaction (ERIC-PCR) and matrix-assisted laser
46 desorption ionization time-of-flight (MALDI-TOF) to assess clonality of *K. pneumoniae* isolates. MALDI
47 typing relies on the microbial proteomic spectra (fingerprints) clustering, whereas ERIC-PCR relies on the

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48 fingerprints due to the presence of multiple copies of conserved consensus sequences in the genomes of
49 bacteria for epidemiological surveillance (9).

50 **Material and Methods:**

51 **Bacterial Isolation and Identification:**

52 A total of 137 non-duplicate MDR *K. pneumoniae* isolates were collected from blood, respiratory, and
53 abscess specimens received in the Department of Medical Microbiology, Postgraduate Institute of Medical
54 Education and Research, Chandigarh, India (PGIMER) from March 2019 to February 2020. Isolates were
55 confirmed as *K. pneumoniae* by MALDI -TOF MS (Bruker Daltoniks, Bremen GmBH Germany). Samples
56 were cultured and incubated overnight at 37°C. Information regarding the type of sample (bronchoalveolar
57 lavage, endotracheal aspirate, sputum, blood, wound swab, and abscess drainage), age and sex of patients
58 and type of admission (inpatient/ward/unit/outpatient) were extracted from the hospital information system.
59 Institutional ethical approval to carry out the study has been obtained (IEC-11/2018-1048).

60 **Antimicrobial susceptibility testing:**

61 Antimicrobial susceptibility of the isolates was performed by the VITEK 2 system against ceftazidime,
62 ceftriaxone, cefepime, ertapenem, imipenem, meropenem, aztreonam, ampicillin-sulbactam, piperacillin-
63 tazobactam, ciprofloxacin, levofloxacin, amikacin, gentamicin, tigecycline and trimethoprim-
64 sulfamethoxazole. The broth microdilution method was performed for minimum inhibitory concentration
65 (MIC) of colistin. *Escherichia coli* ATCC 25922 was used as quality control strain for antibiotic
66 susceptibility tests. Results were interpreted following Clinical and Laboratory Standards Institute (CLSI)
67 guidelines (M-100, Ed 2019) (10). Strains not susceptible to at least one agent in three or more antimicrobial
68 classes were defined as multidrug-resistant and were used in the study.

69 **Detection of genes conferring resistance to carbapenems, β-lactams and mobile colistin resistance-1:**

70 Total DNA of all the isolates of *K. pneumoniae* was extracted using the boiling method and further used to
71 detect the presence of the following genes – bla_{NDM}, bla_{KPC}, bla_{OXA1} and bla_{OXA48}, $bla_{\mathrm{VIM,}}$ $bla_{\mathrm{CTXM\text{-}15}}$, bla_{SHV},
72 bla_{TEM}, and bla_{IMP}. PCR assays were carried out using specific primers as described by Dallenne *et al* (11)

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73 and detection of plasmid mediated colistin resistance was performed using universal primer for CLR5
74 region of *mcr*1 gene . *K. pneumoniae* ATCC 1705 was used as a standard positive control strain for bla_{SHV}
75 and bla_{KPC}. For the other genes, in-house strains known to harbor the genes (and confirmed by targeted
76 Sanger sequencing) were used as positive controls. A non-ESBL producing organism (*E. coli* ATCC 25922)
77 was used as a negative control. Primers used for amplification are listed in supplementary file.

78 **Enterobacterial Repetitive Intergenic Consensus- Polymerase Chain Reaction (ERIC-PCR):**

79 ERIC-PCR technique was carried out in thermocycler (Thermo ABI Veriti^{TM} Biosystems) using ERIC
80 primers-forward: 5′-ATG TAA GCT CCT GGG GAT TCAC-3′ and reverse: 5′-AAG TAA GTG ACT
81 GGG GTG AGC G3′. The PCR protocol consisted of an initial denaturation cycle at 95ºC for 5 min,
82 followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 48ºC for 1 min, extension at 72 ºC
83 for 1 min, and a final cycle of amplification at 72ºC for 10 min. PCR products were loaded on 1.5% agarose
84 gel (Lonza SeaKem LE Agarose) at constant voltage of 70V for one hour, and the banding patterns were
85 visualized under ultraviolet radiation (supplementary file). ERIC patterns were analyzed by online data
86 analysis service of PyElph (12). ERIC profiles were compared using Dice similarity matrix coefficient and
87 clustered by neighbor joining method to prepare the phylogenetic tree. Isolates with two or more different
88 bands in ERIC banding pattern were considered as different ERIC type. The dendrogram was drawn
89 according to the clusters (Fig1).

90 **Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) typing:**

91 Identification and phylogenetic analysis of all the isolates was performed according to the method
92 prescribed by Bruker Daltonik, GmbH. In brief, a single bacterial colony was homogenously smeared onto
93 a target spot on a reusable steel plate (MSP 96 target polished steel BC) using a sterile wooden toothpick.
94 Bacterial components were crystalized by overlaying each spot with 1µl of saturated α-cyano-4-
95 hydroxycinnamic acid matrix solution (10mg/ml conc. of HCCA powder in 50% acetonitrile with 2.5%
96 trifluoroacetic acid). Each spot was air-dried at room temperature. Mass spectra of all the isolates were
97 acquired automatically using default identification settings of FlexControl Software with linear positive

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98 mode, mass range 2-20kDa. The final spectrum was the sum of 20 single spectra, each obtained by 200
99 laser shots on random target spot positions. The raw spectrum obtained were processed and classified with
100 the help of MALDI BioTyper software, version 3.1. The standard interpretative criteria for Bruker were
101 applied. A phylogenetic tree (Fig 2) was constructed from main spectrum profiles of all the isolates using
102 FlexControl software (version 3.4.127.0) and BioTyper software (version 3.1).

103 **Discriminatory index (D):**

104 The discriminatory indices (D) of MALDI-TOF and ERIC-PCR were calculated based on Simpson's Index
105 of Diversity formula (13) as below:

106 $D=1-(\sum n(n-1)/N(N-1))$

107 Where D is diversity index, N is the total number of individual strains in the sample population and n is the
108 number of isolates of a particular type/cluster. Simpson's index of diversity ranges from 0.0 to 1.0 where
109 value equal to 1.0 is highly discriminatory, and a value of 0.0 represents that all the isolates are of an
110 identical type.

111

112 **Results**

113 **Clinical isolates.** Total 137 multidrug resistant *K. pneumoniae* clinical isolates from endotracheal aspirates,
114 sputum and bronchoalveolar lavage (n=30), wound pus (n=62), body fluids, CSF (n= 7), and blood (n=38)
115 were included (Fig 3). Lactose fermenting colonies on MacConkey agar were further identified by MALDI-
116 TOF MS.

117 **Polymerase chain reaction-based detection of extended spectrum beta-lactamases and**
118 **carbapenemase-encoding genes:**

119 Among the various genes tested, all the isolates expressed at least two antimicrobial resistance genes, 95%
120 isolates expressed bla_{TEM} followed by $bla_{\text{CTXM-15}}$ (~88%). Amongst the carbapenemase genes tested,
121 maximum expression was for bla_{OXA} (~88%) followed by bla_{NDM} (~54%) as indicated in Table1 and Fig 4.

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122 **Table 1: PCR based ESBL and carbapenemase gene expression analysis**

Genes tested	Positive	Percentage
TEM	129	94.85
SHV	105	77.21
CTXM-15	120	88.24
KPC	72	52.94
OXA1 and 48	120	88.24
VIM	70	51.47
NDM-1	73	53.68
IMP	28	20.59

123

124 **Molecular fingerprinting by ERIC-PCR:**

125 The number of bands varied from 1 to 11 with the size ranging from 100 bp to more than 1.5kb. A total of
126 40 different ERIC profiles (E-types) were observed designated as E1 to E40. Out of 137 isolates, total 44
127 isolates belonged to E1, 8 isolates to E2,17 isolates to E11, 12 isolates to E19 type, 2 each to E5, E6, E12,
128 E13, E16, E31, E33, E24, E10=6 isolates, E7 and E35=4 each, E21=3 and other remaining 24 isolates
129 showed a unique pattern. To further investigate probable outbreak of MDR *K. pneumoniae* infection, ERIC
130 typing data was mapped in relation to hospital unit/ward (Fig 5). Details of the same has been provided in
131 the supplementary file.

132 **Matrix assisted laser desorption ionization time of flight analysis:** Isolates were allotted into 6 groups
133 *i.e.*, G1to G6 by MALDI-TOF analysis. G1=3 isolates, G2=10 isolates, G3=26 isolates, G4=1 isolate, G5=4
134 isolates and G6=93 isolates.

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135 **Discriminatory potential of ERIC-PCR and MALDI-TOF in typing of *K. pneumoniae* isolates:** The
136 Simpson's Diversity Index was calculated for ERIC-PCR, and MALDI-TOF analysis as depicted in the
137 table below. ERIC -PCR was found to be more discriminatory (D=0.8704) for clonal relatedness and was
138 a better phylotyping tool in comparison to MALDI-TOF typing (D=0.5001).

Typing method	**Simpson's Discriminatory index**
ERIC-PCR	0.8704
MALDI-TOF	0.5001

139

140 **Discussion**

141 Given the high prevalence of MDR *K. pneumoniae*, it is essential to understand and establish the clonal
142 relatedness among isolates to prevent and control *K. pneumoniae* outbreaks in a healthcare setting. Various
143 molecular typing methods such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis
144 (PFGE), plasmid typing, antibiogram typing and ribotyping have been used for epidemiological and
145 surveillance studies. However, many of these are expensive, labor intensive and time-consuming. Methods
146 such as palindromic repetitive element-based ERIC-PCR and proteomic signature based MALDI-TOF are
147 quick, reliable, and cost-effective techniques for molecular typing of the Enterobacteriaceae family. We
148 therefore studied these tools to determine the transmission dynamics of MDR *K. pneumoniae* in hospital
149 settings.

150 *Klebsiella pneumoniae* is a problem pathogen with a high incidence of multidrug resistant strains. To study
151 the epidemiological relatedness among the MDR *K. pneumoniae* isolates and to find out the possibility of
152 any plausible outbreak, we designed this study comparing two methods for typing, one based on the
153 genotype (ERIC-PCR) and another on the basis of protein profiling (MALDI-TOF). The importance of
154 molecular typing of MDR- *K. pneumoniae* by these methods is in strengthening the epidemiological

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155 surveillance and to recognize the clonal spread in a rapid and cost-effective manner in comparison to WGS
156 based surveillance. We analyzed a total of 137 isolates and found the genotyping-based method to be more
157 robust and discriminatory than MALDI-TOF as indicated by the Simpson's diversity index value (for
158 ERIC-PCR, D=0.8704).

159 In this study, 45% isolates were related to wound pus followed by 28% from blood and 22% from
160 respiratory specimens (Fig 3). ERIC-PCR devised phylogenetic group E1 was most prevalent (29%) and
161 represented a dominant clone, followed by E11 (12.4%) and E19 (8.7%). Out of the 40 isolates from E1
162 phylogenetic group 13 isolates were collected from general outpatient department (OPD), 7 from AGE-
163 ICU and rest of the isolates from other indoor and outpatient hospital units/wards. Similarly, isolates
164 belonging to other ERIC phylogenetic groups were sampled from different indoor and outpatient hospital
165 units/wards. Our observations revealed that no outbreak or nosocomial clustering happened during the
166 designated period of sampling.
167 MALDI typing showed a group G6 (68%) was most prevalent followed by G3 (19%). The unit/ward data
168 also did not show any correlation with MALDI groups; supporting our observation from ERIC-PCR data.
169 Both the techniques indicated the convergent result of no likely outbreak/nosocomial spread of MDR *K.*
170 *pneumoniae* isolates during the study period. Although both the tools provided convergent outcomes (Fig
171 6), ERIC-PCR was a better tool with Simpson's diversity Index (D=0.8704) approaching close to numerical
172 value of one. Of the 137 MDR *K. pneumoniae* isolates used in this study, ERIC-PCR revealed 40 (E1-E40)
173 and MALDI-TOF revealed 6 (G1-G6) distinct groups. Among the isolates that were classified as very
174 closely related based on their MALDI-TOF dendrogram, the ERIC-PCR banding patterns showed lack of
175 genetic relatedness, thereby highlighting the difference in clustering the isolates based on genomic versus
176 proteomic signatures. The large number of serotypes in this species could also explain this genetic diversity
177 highlighted by the ERIC-PCR genotypic analysis. Similar to other studies, in our study the clustering
178 pattern by MALDI-TOF was different compared to that seen with ERIC-PCR based techniques (14,15).
179 This might be because of the fact that phenotype expressed is not always the true representative of genotype.

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180 In this study, the discriminatory indices for ERIC-PCR and MALDI-TOF were performed. Comparison
181 between these two methods revealed the higher discrimination index for ERIC-PCR than MALDI -TOF,
182 but results were not comparable. ERIC-PCR banding pattern gave a meaningful clustering with a Simpson's
183 Discriminatory index of 0.8704. Proteomics based MALDI-TOF clustering showed that the *Klebsiella*
184 *pneumoniae* isolates tested were heterogeneous and clonal relatedness could not be depicted as Simpson's
185 Discriminatory index was 0.5001. Similar to our study Rim et al found MALDI-TOF to have
186 low/insufficient discriminatory power to determine the relatedness of MDR-*Acinetobacter baumannii*
187 isolates (16). A recent study by Purighalla S et al., have the supporting results indicating ERIC being more
188 reproducible and better tool than MALDI-TOF to determine the relatedness of nosocomial *K. pneumoniae*
189 isolates (14) though with routine use of MALDI for microbial identification, it is also being used for
190 outbreak investigations with no added cost for effective interventions (17,18). Although MALDI-TOF is a
191 quick and easy technique and can speed up the infection control measures to prevent further outbreak (19),
192 it does not fit in every situation for perfect discrimination (20). Maskit Bar-Meir also supported the
193 integration of MALDI-TOF-MS, primarily for species identification and only secondarily for
194 epidemiological typing (21). Various studies show that MALDI biotyper has limited ability to provide
195 protein fingerprinting because it targets ribosomal proteins in the limited range of 2-20 kD which is
196 adequate for identification but shows restricted ability to differentiate isolates to the level of clonal complex.
197 Also, there are scopes of improvement for MALDI-TOF MS as a tool for biotyping as its performance also
198 varies based on modification of the growth conditions and extraction method (21, 22).

199 **Conclusion:** Although genomic tools have good discriminatory power but are costly, labour intensive and
200 demand expert handling. ERIC -PCR profiles provide good discriminatory power to find out the clonal
201 relatedness to describe any likely epidemiological outbreak for MDR *K. pneumoniae* isolates. This
202 phylotyping tool is easy, fast, cost effective and can be easily adapted to high throughput analysis of isolates
203 for meaningful clustering.

204 **Conflicts of Interest:** All the authors declare that there are no conflicts of interest regarding the publication
205 of this article.

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208 **Author's role:**

209 JK: Conceptualization, data curation/analysis and visualization, Writing -Original Draft Preparation,
210 Conducting experimentation, Validation.

211 SK: Data curation/analysis and visualization, manuscript writing-reviewing/editing, validation.

212 MK: Sample collection and processing.

213 SR: Sample collection, conducting experimentation, reviewing.

214 AA: Project administration, Reviewing and editing the manuscript, Data validation, Supervision.

215 MB: Reviewing and editing the manuscript, supervision.

216 KW: Reviewing the manuscript, Data validation, supervision

217 PR: Supervision, Project administration, Reviewing and editing the manuscript, Resources.

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297 **Legends to figures :**

298 **Fig 1:** Dendrogram generated with neighbor end joining and the UPGMA clustering methods, showing the
299 genetic similarity among *K. pneumoniae* isolates by enterobacterial repetitive intergenic consensus (ERIC)
300 genotyping.

301 **Fig 2: MALDI-TOF dendrogram-** phylogenetic tree was constructed from main spectrum profiles of all
302 the isolates using Flexcontrol software (version 3.4.127.0) and Biotyper software (version 3.1). Colour
303 coding indicates the MALDI types of the isolates.

304 **Fig 3:** Distribution of clinical isolates as per the origin of sample collection.

305 **Fig 4**: Heat map representing the carbapenemases/ESBL gene distribution with respect to isolates from
306 different hospital units/wards.

307 **Fig 5:** ERIC typing of the isolates as per the sample collection site/ward/unit.

308 **Fig 6:** Convergent results with ERIC and MALDI-TOF as typing tools for relatedness of clinical isolates.
309 As depicted in the bar graph ERIC has better discriminatory power for large sample size in comparison to
310 MALDI-TOF.

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Figure 1

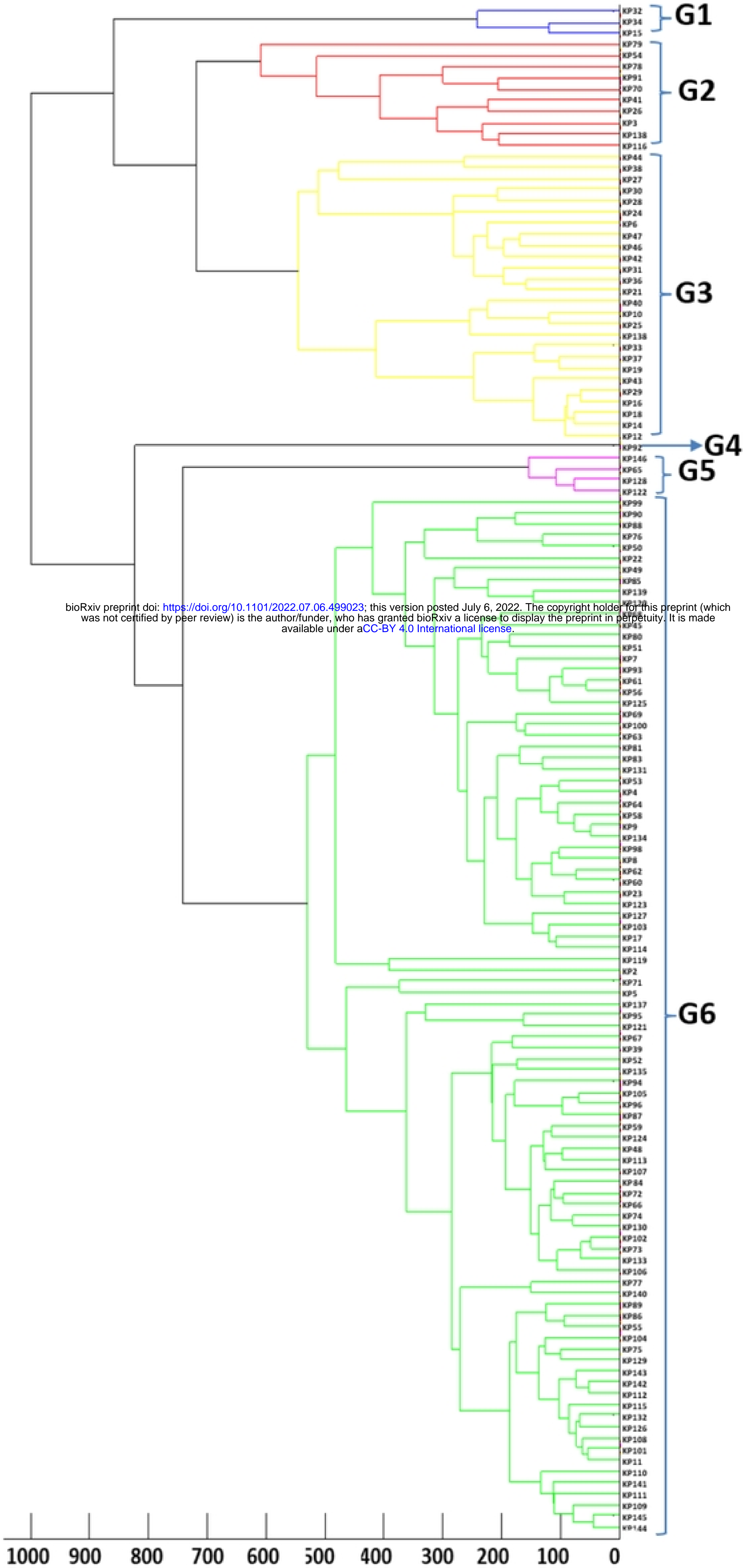

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Figure 2

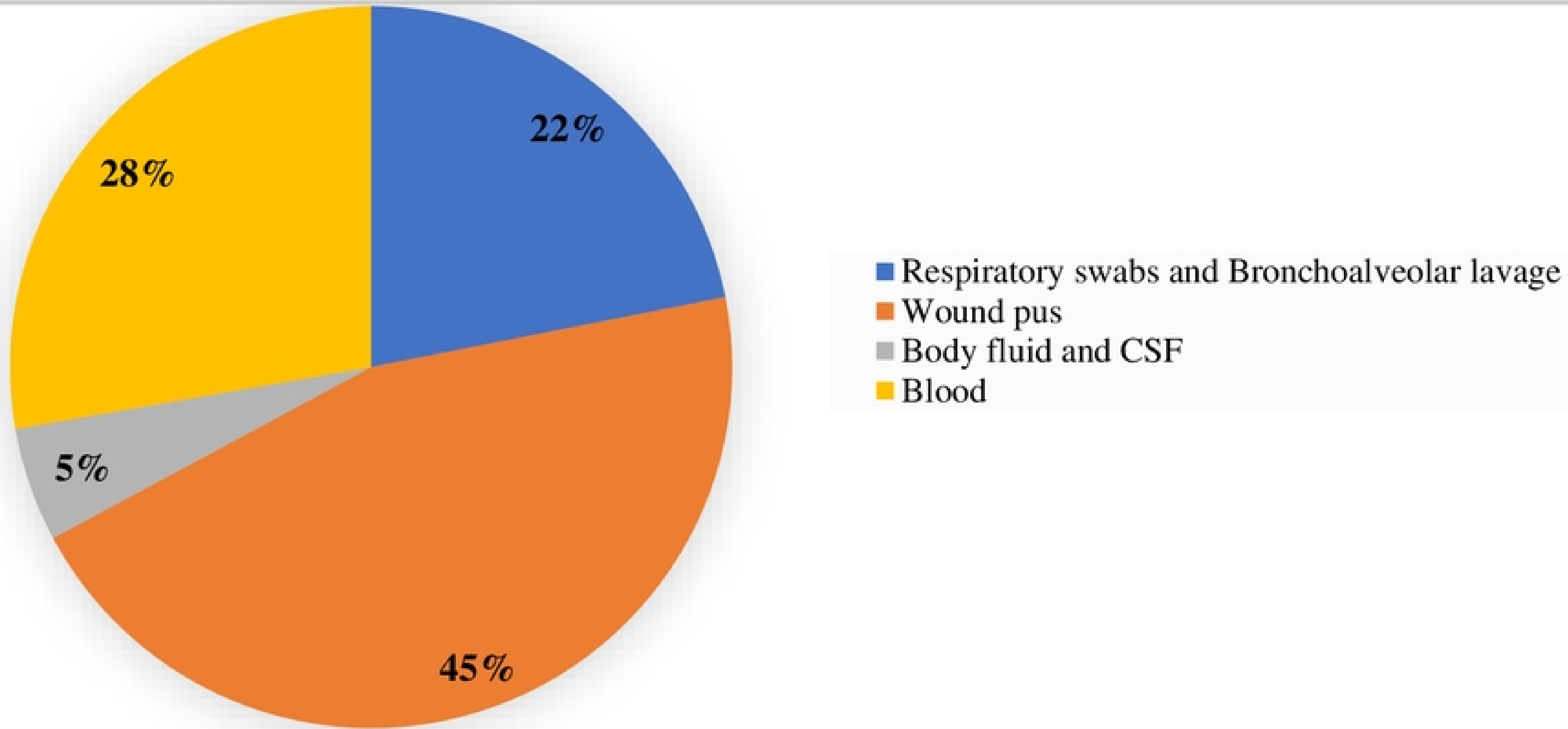

Figure 3

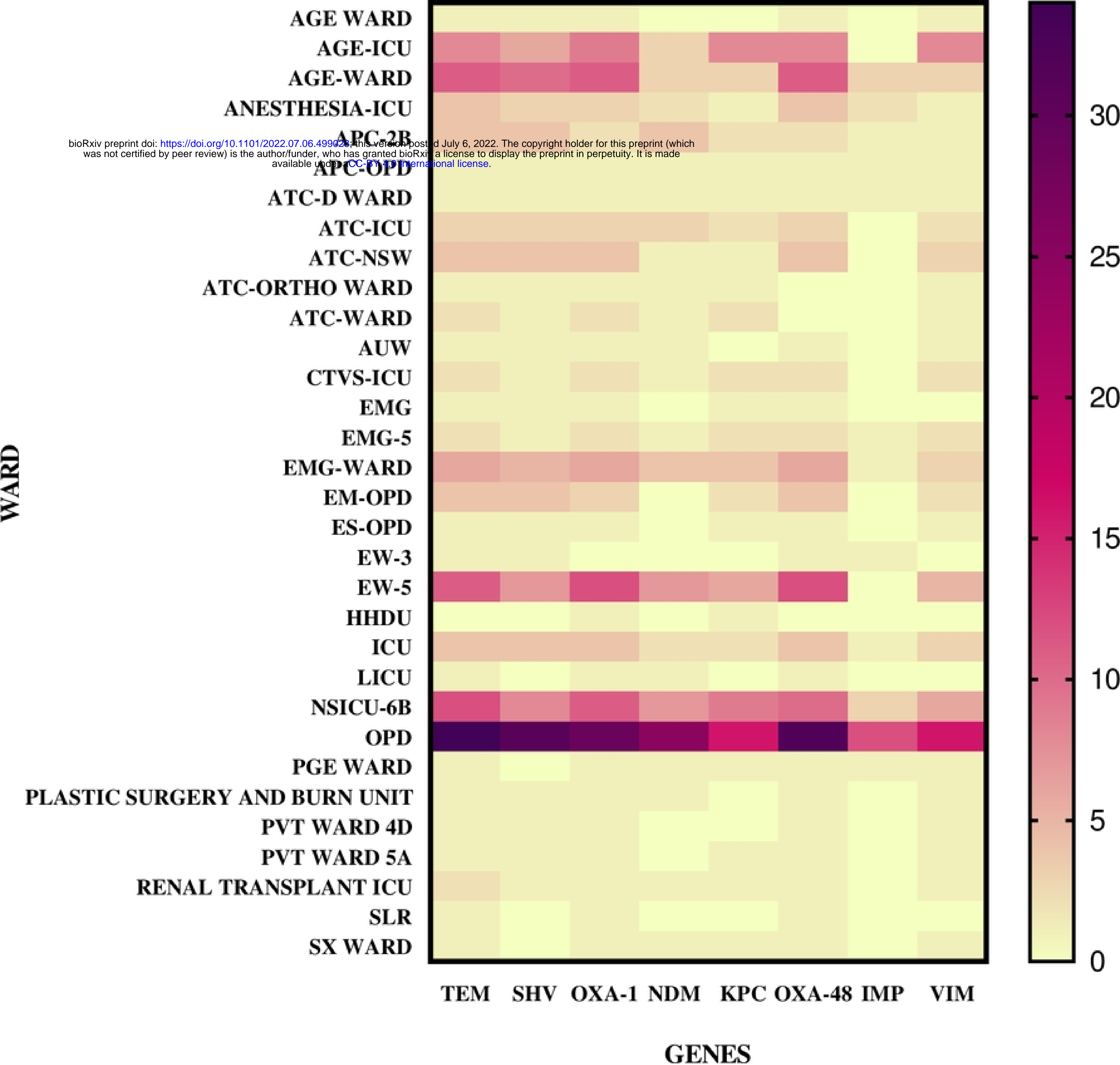

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Figure 4

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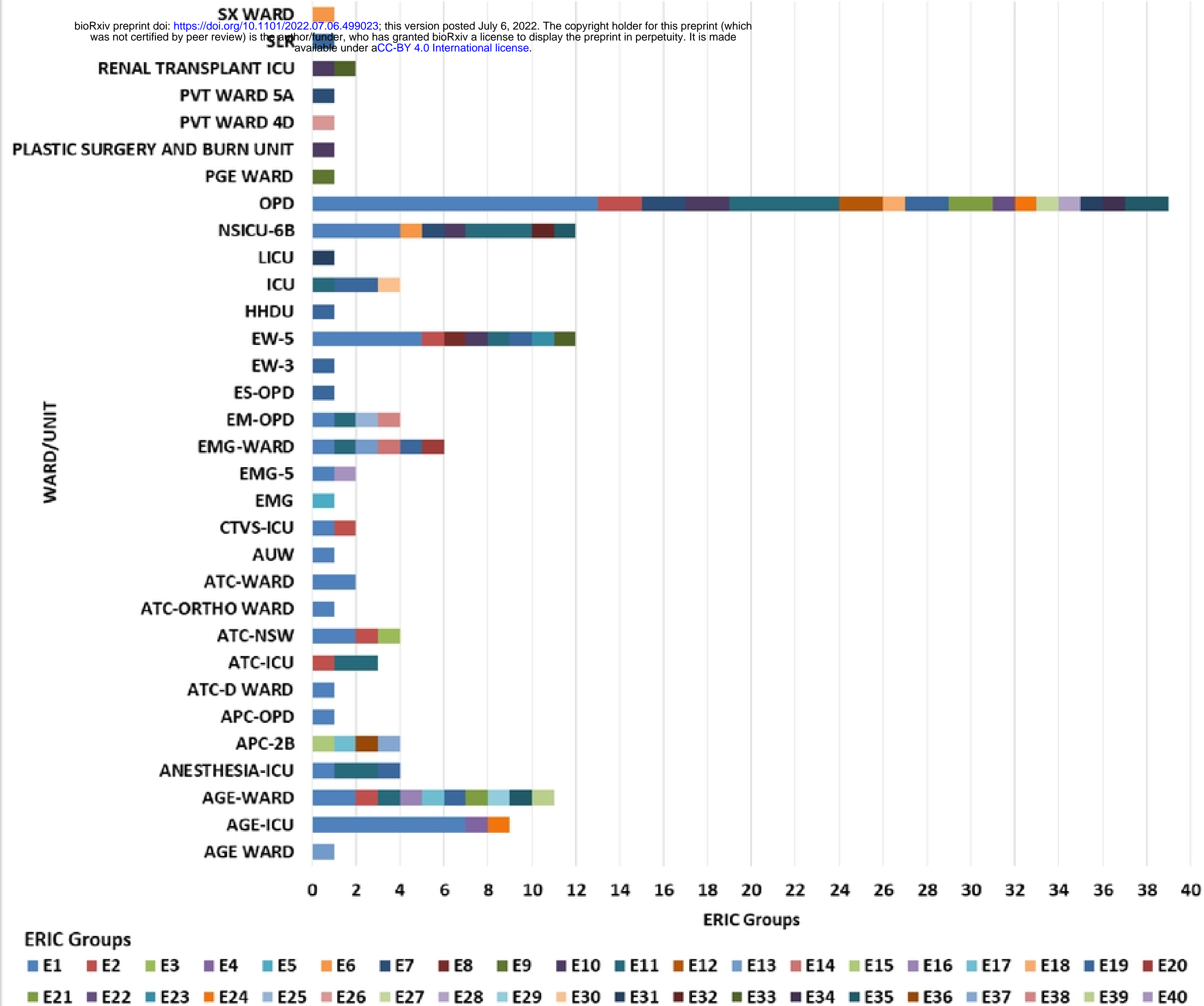

Figure 5

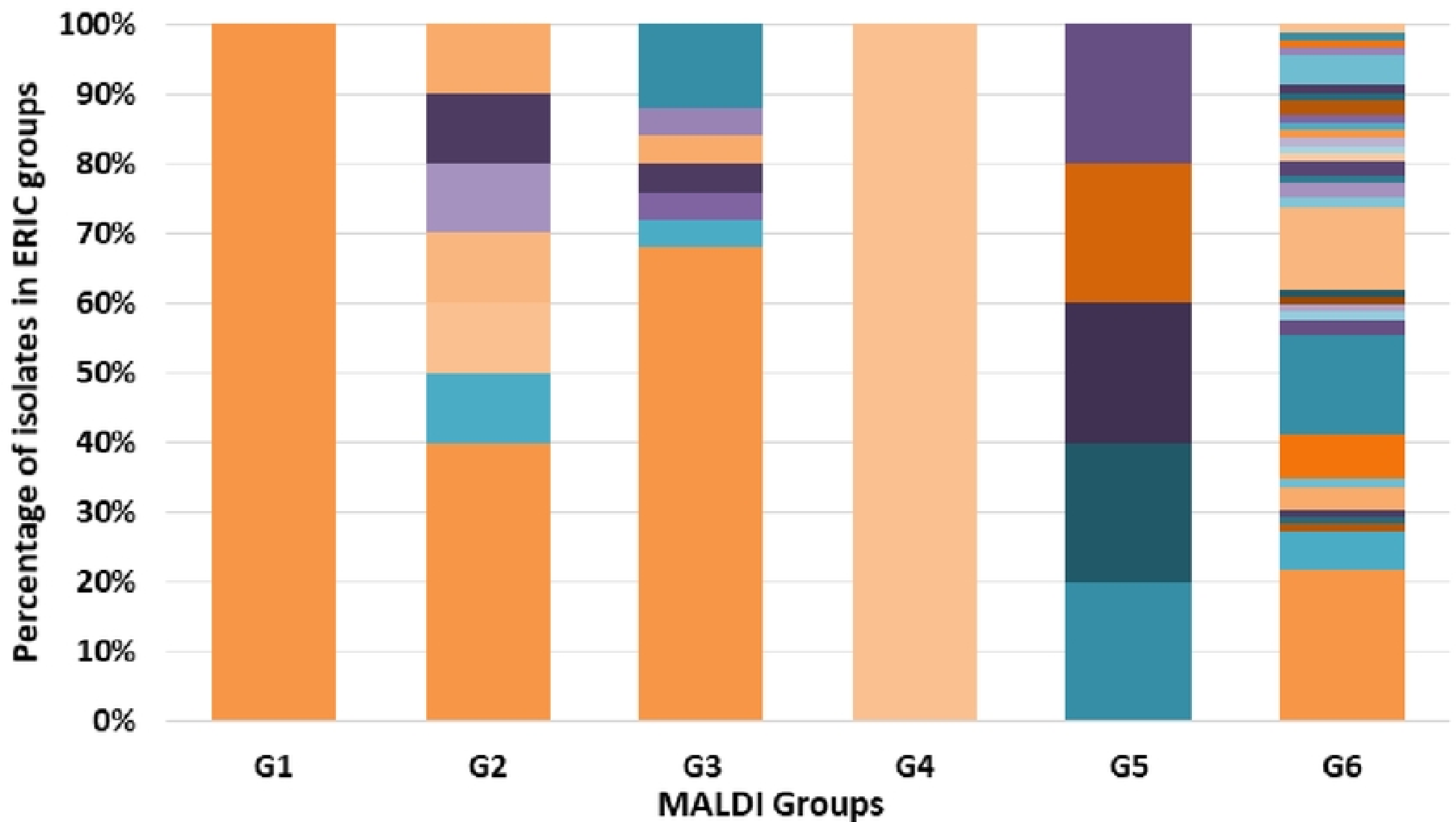

Figure 6