Revealing virulence potential of clinical and environmental *Aspergillus fumigatus* isolates using Whole-genome sequencing

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Abstract

Aspergillus fumigatus is an opportunistic airborne pathogen and the most common causative agent of human fungal infections. A restricted number of virulence factors have been described but none of them lead to a differentiation of the virulence level among different strains. In this study, we analyzed the whole-genome sequence of a set of *A. fumigatus* isolates from clinical and environmental origin to compare their genomes and to determine their virulence profiles. For this purpose, a database containing 244 genes known to be associated with virulence was built. The genes were classified according to their biological function into factors involved in thermotolerance, resistance to immune responses, cell wall structure, toxins and secondary metabolites, allergens, nutrient uptake and signaling and regulation. No difference in virulence profiles was found between clinical isolates causing an infection and a colonizing clinical isolate, nor between isolates from clinical and environmental origin. We observed the presence of genetic repetitive elements located next to virulence related gene groups, which could potentially influence their regulation. In conclusion, our genomic analysis reveals that *A. fumigatus*, independently of their source of isolation, are potentially pathogenic at the genomic level, which may lead to fatal infections in vulnerable patients. However, other determinants such as genetic variations in virulence related genes and host-pathogen interactions most likely influence *A. fumigatus* pathogenicity and further studies should be performed.

Importance

Aspergillus spp. infections are among the most clinically relevant fungal infections also presenting treatment difficulties due to increasing antifungal resistance. The lack of key virulence factors and a broad genomic diversity complicates the development of targeted diagnosis and novel treatment strategies. A widely spread variability in virulence has been reported for experimental, clinical and environmental isolates. Here we provide supporting evidence that members of this species are fully capable of establishing an infection in immunosuppressed hosts according to their virulence content at the genomic level. Due to the possible clinical complications, studies are urgently required linking strain's virulent phenotype with the genotype to better understand the virulence activation of this important fungal pathogen.

72 **Introduction**

73 *Aspergillus fumigatus* (*A. fumigatus*) is an opportunistic fungal pathogen that poses one of the
74 major threats to immunocompromised individuals in the clinic. High risk patients include
75 neutropenic patients, hematopoietic stem cell transplant recipients, patients receiving a
76 prolonged steroid treatment and critically-ill patient in the intensive care unit (ICU) with
77 chronic obstructive pulmonary disease (COPD), liver cirrhosis, viral infections or microbial
78 sepsis (1-3). In the context of an impaired immune function, inhaled airborne spores of *A.*
79 *fumigatus* will not be effectively eliminated and will remain in human airways to cause a
80 range of infections that include allergic bronchopulmonary aspergillosis (ABPA), aspergilloma
81 (chronic aspergillosis) and invasive aspergillosis (IA) (1,4). IA is the most serious infection,
82 with a global prevalence of 250,000 cases per year with mortality rates up to 90-95% (5,6).
83 In addition to the increasing burden of patients with impaired immunity (1), another major
84 challenge is the treatment of these fungal infections due to the antifungal resistance to
85 triazoles, the most indicated drugs against *Aspergillus* species infections. Resistance is
86 characterized by the presence of a point mutation (L98H) in the azole target *Cyp51A* and a 34-
87 base pair (bp) tandem repeat (TR34) in its promoter region (7) and the most common cause
88 of resistance acquisition is the widespread azole-based fungicide use against fungal plant
89 pathogens in the agricultural practice (7-9).

90 In order to overcome the therapeutic challenges and threats posed by *A. fumigatus* infections,
91 there is a need to further understand the mechanisms of adaptability and infection of the
92 fungus to develop better and early diagnostic tools and uncover novel therapeutic strategies.
93 The virulence of *A. fumigatus* is multifactorial, a trait that has been developed by the fungus as
94 a need to survive the encountered selective pressures in decaying vegetation (10). Whole-
95 genome and transcriptome analysis have allowed the discovery and study of new components
96 of *A. fumigatus* biology and pathogenesis, providing a better understanding of the genetic
97 content of *Aspergillus spp*. Genomic analyses identified that *A. fumigatus* contains 8.5% of
98 lineage-specific (LS) genes with accessory functions for carbohydrate and amino acid
99 metabolism, transport, detoxification, or secondary metabolite biosynthesis, suggesting this
100 microorganism has particular genetic determinants that can facilitate an *in vivo* infection (11).
101 Nevertheless, the study of *A. fumigatus* virulence has been hampered by the lack of a standard
102 wild-type (WT) strain, next to a broad isolate-dependent variability in virulence as shown in
103 murine infection models of IA (12). *A. fumigatus* isolates can be divided in three different
104 categories depending on the source of isolation: 1) environmental, obtained from decaying

105 vegetation, air sampling, crops, etc.; 2) clinical, originally found in patient samples, and 3)
106 experimental, which refers to isolates that were first obtained from a patient setting but are
107 now used as reference strains by many research groups (i.e. Af293 or CEA10). Some
108 experimental infection studies have reported clinical isolates to be more virulent than
109 environmental isolates (13-15). Moreover, two environmental isolates retrieved from an
110 International Space Station were more virulent than the experimental strains Af293 and
111 CEA10 (16). Additionally, an in-host study that analyzed the microevolution of 13 isogenic
112 isolates of *A. fumigatus* obtained from the same patient over a period of 2 years, reported both
113 increases and decreases in virulence among the isolates (17). The latter, most likely as a result
114 of the adaptation of this microorganism to the human niche to allow its persistence (17).
115 These different observations highlight the need to recognize the intraspecies genotypic and
116 phenotypic variety among *A. fumigatus* populations, as this could determine the progression
117 and fulminant outcome of diseases produced by this fungus.
118 To increase the knowledge on molecular factors contributing to the development of diseases
119 caused by *A. fumigatus*, the present study aimed to determine the underlying genetic traits
120 that characterize a virulent strain. We evaluated if differences in the afore mentioned strain-
121 specific virulence can be explained through abundance of virulence factors at the genomic
122 level, with special interest in clinical isolates obtained from patients with different clinical
123 outcomes. A database containing 244 *A. fumigatus* virulence related genes (VRGs), reported
124 by different studies, was created and used to define the pathogenic potential of investigated
125 isolates. In total nine *A. fumigatus* sequences were used in our study: two experimental, five
126 clinical and two environmental isolates. Among these isolates, the whole-genome sequences
127 of three clinical isolates and one experimental strain B5233 were generated at the University
128 Medical Center Groningen (UMCG), and further used for strain genotyping and to perform a
129 comparative genomic analysis to identify genomic differences.
130
131 **Results**
132 **Virulence related genes screening showed that all *A. fumigatus* isolates included in this**
133 **study are potentially pathogenic.** The genome sequences of nine *A. fumigatus* isolates
134 (Table 1) were screened for the presence of particular VRGs using our in-house database (see
135 Table S2). We identified the presence of all 244 VRGs (>90% coverage and >90% identity) in
136 the genome of seven isolates P1MR, P1MS, P2CS, Af293, 12-7505054, 08-19-02-30 and 08-19-
137 02-46. In addition, 243 genes were present in the genomes of B5233 and 08-12-12-13, and

138 both isolates lacked the *Afu5g12720* gene. This gene codes for a putative ABC transporter and
139 is a member of the Biosynthetic gene cluster 17 (BGC17), consisting of in total 10 genes,
140 described by Bignell *et al* (18). The product of this BGC is a non-ribosomal peptide synthetase
141 (NRPS), which is thought to be structural (19). However, no clear link between this ABC
142 transporter and the function of this NRPS has been described before, thus it is not known how
143 its absence could affect the overall function of this cluster and its possible role in virulence.
144 **TRESP genotyping identified a distinct genetic background of the isolates.** Out of four
145 investigated isolates, two were isolated from the same patient suffering from an influenza A
146 H1N1 infection with IA (Figure 1). Given that P1MR, a resistant strain, was isolated nine days
147 after a susceptible isolate P1MS was obtained, the question arose as to whether these isolates
148 were genetically related and if the resistant phenotype developed after azole treatment. To
149 determine their genetic relatedness, we used the recently described TRESP method that
150 identifies the alleles of specific tandem repeats in the exon sequence of surface proteins CSP,
151 MP2 and CFEM (Table 2) (20). The four isolates presented different allelic combinations and
152 thus, different TRESP genotypes, P1MS and P1MR having t03m1.1c08A and t11m1.1c09
153 TRESP genotypes, respectively. In this study, CSP alleles appeared to best differentiate the
154 isolates (Table 2).
155 **Comparative genomics illustrates differences in the genome structure.** The genomes of
156 our four isolates were compared to *A. fumigatus* Af293 chromosomes. The compared genomic
157 sequences of the 8 chromosomes are displayed in Figure 2, in which investigated VRGs
158 locations are highlighted in yellow. Small deletions (100 kpb) were observed at the end of
159 chromosomes 5 and 6, and large deletions (>300 kbp) were identified at the beginning of
160 chromosome 1 and at the end of chromosome 7. Multiple small deletions and large-scale
161 deletions in *A. fumigatus* genomes have been reported previously, and particularly these
162 >300kbp large-scale deletions were previously described in chromosome 1 (21, 22) and
163 chromosome 7 (21). A region with a high dissimilarly with respect to the reference Af293
164 between 1,698 kbp and 2,058 kbp is observed in chromosome 7 for all the isolates, where
165 only the P2CS isolate had a certain degree of similarity (Figure 2). In addition, sequence gaps
166 with no assigned CDS, (indicated by a red line in Figure 2), represent putative centromeres in
167 all chromosomes and in the case of chromosome 4, a region of ribosomal DNA (represented
168 by a dark blue line) (11). Notably, repeat rich sequence areas were identified in
169 chromosomes 1, 2, 4, 6 and 8. These areas can be seen in Figure 2 by the alignment of many
170 small contigs which coincides with a low GC content. In the case of chromosome 4, a group of

171 virulence genes appears to be flanked by these repetitive regions on both sides, whereas some
172 groups are only flanked on one side as depicted in chromosomes 6 and 8.
173 **Genomic variability among the fungal genomes.** Variant calling using as reference the *A.*
174 *fumigatus* Af293 genome, identified a total number of 68,352; 48,590; 56,362 and 56,422
175 variants in the genome of B5233, P1MS, P1MR and P2CS isolates, respectively (Table 3). High
176 and moderate impact variants were retrieved, and their predicted effect is displayed in Table
177 S3. Among variations with a predicted moderate- and high-impact, a high number of missense
178 variants, ranging from 9,804 to 12,067, was identified. Single nucleotide polymorphism (SNP)
179 analysis in VRGs with respect to reference Af293 revealed the presence of a range of 1,015 –
180 1,122 SNPs in all the analyzed isolates (Table 3). Examples of some variants present in the
181 VRGs are listed in Table 4 and a more detailed description is given in supplementary Table S4.
182 No clear pattern of variant distribution was found regarding the source the isolates. Rather,
183 we observed some cases where all isolates had common variants as demonstrated in genes
184 *thtA, sidC* and *msdS.* Genes associated to resistance to the immune response *rodB*, *cat1* and
185 *afpmt2* had only one or no variants suggesting they are highly conserved genes. The *gliZ gene*
186 required for the regulation of gliotoxin and the *gli* cluster, and the *sidC* gene, with an
187 important role in iron acquisition, are examples of genes with several variants.
188 Additionally, a comparative analysis between P1MS and P1MR isolates from the same patient
189 was performed and 45,335 variants were found, corresponding to 38,319 SNPs, 868 multiple
190 nucleotide polymorphisms (MNPs), 1,768 insertions, 1,842 deletions and 2,538 complex
191 (combination of a SNPs and MNPs).
192
193 **Discussion**
194 *A. fumigatus* is a major fungal opportunistic pathogen capable of causing chronic and deadly
195 invasive infections. The development of an infection seems to be primarily determined by the
196 host immune status. Here, we performed a genomic analysis to investigate the role of the
197 pathogen in the development and progression of the infection. We hypothesized that *A.*
198 *fumigatus* isolates recovered from a patient who died after an infection with influenza A H1N1
199 and IA and, an isolate from a patient with human immunodeficiency virus (HIV) and COPD
200 with no reported Aspergillus infection, would reveal a distinct virulence profile. In addition to
201 our clinical isolates, we studied the known virulent *A. fumigatus* experimental strain B5233
202 and five unrelated isolates available in public databases with different sources of isolation
203 (Table 1). Our analysis identified the presence of 244 VRGs in all *A. fumigatus* isolates tested,

204 with the exception of *Afu5g12720* gene in B5233 and 08-12-12-13, indicating all isolates have
205 the potential to be or become pathogenic. Thus, differences in the microorganisms' capacity to
206 cause damage most likely do not only rely on the presence or absence of virulence factors at
207 the genomic level of *A. fumigatus*. Moreover, a high variability in the studied *A. fumigatus*
208 genomes, reflects the enormous capacity of adaptation of the fungus to different
209 environments.
210 The only gene amongst the 244 VRGs included in our in-house database, not found in the
211 isolates B5233 and 08-12-12-13 was *Afu5g12720*. Interestingly, this gene was reported to be
212 absent in 21 out of 66 *A. fumigatus* samples in a population genomics study that investigated
213 the genomic variation of secondary metabolites in this species (23). This gene is a member of
214 the BGC 17, and its absence could have a functional impact on the synthesis of the final
215 product of this cluster, a NRPS, which is thought to have a structural function (19).
216 *Afu5g12720* codes for an ABC transporter located in the BGC17 with another 9 genes (18) and
217 was curiously absent in B5233, a strain that has been described as highly virulent. It would be
218 interesting to further study the link between the lack of this gene and a possible increase in
219 virulence, since disruption of another gene member included in this BGC17, *pes3*, resulted in a
220 hypervirulent strain (19).
221 Additional changes in the genome structure of our isolates were found in our comparative
222 genomic analysis. We identified deleted segments in relation to the reference that were
223 located at the beginning of chromosome 1 and at the end of chromosomes 5, 6 and 7.
224 Fedorova *et al*, identified these subtelomeric regions to be enriched for the presence of
225 pseudogenes, transposons and other repetitive elements, and corresponding to areas that
226 contain genes specific to the *A. fumigatus* species. (11) It was hypothesized that these genes
227 have most likely evolved from big duplication and diversification events and not horizontal
228 gene transfer. (11) It seems isolates B5233, P1MS and P1MR also do not have these genes. Our
229 hypothesis is that these segments, are insertion-prone regions that are contributing to the
230 diversification of the species.
231 Nucleotide variant analysis of our four isolates, identified a total number of variations ranging
232 from 48,590 to 68,352 compared to reference strain Af293. This range fits in the genetic
233 diversity for *A. fumigatus* reported previously and determined in 95 sequences ranging from
234 36 - 72,000 SNPs (16). The large number of identified variants and differences in the genome
235 structure displays a broad genetic diversity in the studied isolates. This diversity is
236 hypothesized to directly influence the fungus virulence by allowing an adaptation to an in-

237 host environment, the evasion of the host immune system and the acquisition of antifungal
238 resistance (17,24,25,27). The presence of SNPs in the VRGs of the clinical isolates, particularly
239 those to be predicted to have a high impact, could be of major influence for the virulence of
240 the isolates. However, in this study we could not link the presence of genetic changes in VRGs
241 to isolates with a common origin of isolation. In addition, some repetitive elements were
242 located on the sides of some groups of VRG as exemplified on chromosomes 6 and 8. It is
243 possible they could play a role in how some of these genes are expressed, since these elements
244 are recognized to shape the genomes of fungi (28). Follow-up studies using RNA sequencing
245 could help to elucidate how and under what circumstances these virulence genes are
246 expressed, as well as to determine the impact of genomic variations on expression levels.
247 Subsequent infection model studies could be used to correlate these genomic variations and
248 changes with specific pathogenic phenotypes.
249 The genome sequence of isolates P1MS and P1MR differed by 45,335 variants and they were
250 confirmed to have different genotypes, supporting the hypothesis that resistance did not
251 develop from the initial susceptible isolate. It is also unlikely that in a period of nine days the
252 susceptible isolate would have been able to mutate to acquire azole resistance since the
253 median time of development of azole resistance has been reported to be 4 months (29).
254 Moreover, gain of the resistant phenotype within the host is observed in chronic infections
255 whereas acquisition of resistance during IA continues to be unreported (24). In a similar case
256 of post-influenza aspergillosis, four *A. fumigatus* isolates were obtained from a patient that
257 received an allogeneic stem cell transplant and developed IA after the influenza virus infection
258 which was initially treated with voriconazole (30). The patient passed away 4 months later.
259 The first three isolates were susceptible to azole treatment but the last one, demonstrated
260 triazole-resistance. It was confirmed that the resistant isolate was different from the first
261 isolates by STR*Af* microsatellite genotyping (30). It is likely that the resistant phenotype of the
262 resistant isolate in both our study and the post-influenza study (30), was of environmental
263 origin and that this isolate had coexisted with the susceptible isolates in a mixed population
264 that was not detected during the first sampling. Treatment with voriconazole most probably
265 eradicated the initial susceptible strain and through selective pressure, allowed the resistant
266 *A. fumigatus* strains to persist in the patient's airways. Because of this possibility, a change of
267 practice regarding *A. fumigatus* isolation has been applied at the diagnostics laboratory at the
268 UMCG where antifungal susceptibility testing is now applied to at least five colonies obtained
269 from a single respiratory sample. Influenza virus infection has been recently described as an

270 independent risk factor for invasive pulmonary aspergillosis and, therefore, extreme care for
271 patients admitted into the ICU with a severe influenza virus infection is advised (31).
272 The use of TRESP genotyping in this study identified the isolates to be genetically different.
273 This approach was easy and accessible and only required the whole-genome sequence of the
274 isolates, in contrast to other traditional typing methods with much lower discriminatory
275 power (MLST) (32), laborious microsatellite determination (STR*Af*) (33), and the most novel
276 whole-genome SNP based typing method which is highly dependent on the quality of
277 sequencing, variant calling parameters and selection of a genetically close reference strain
278 (21).
279 We show evidence that supports the observation that all fungi have the ability to cause
280 disease and that members of the *A. fumigatus* species lack the sophisticated virulence factors,
281 commonly used to describe differences in virulence in species of the bacterial kingdom, that
282 could explain differences in their pathogenic traits (25,34). In order to define what makes a
283 virulent *A. fumigatus* isolate many researchers have attempted characterizing different
284 aspects of the fungus: differences in the colonial and spore color phenotype (25), the strain-
285 dependent immunomodulatory properties induced in the host (25), the clinical or
286 environmental source of the isolate (13, 25,26), the strains ability to adapt and grow in
287 stressful conditions like low oxygen microenvironments where hypoxia fitness was strongly
288 correlated with an increase in virulence (26), and the ability of the fungus to adjust its gene
289 expression to survive in different immunosuppressive conditions inside the host (3). These
290 are all aspects that influence how fit a strain will be to produce an infection and further
291 research on the virulence of this microorganism should take all these aspects into
292 consideration when characterizing an experimental and/or clinical strain. The combined
293 results of these studies could be used to explore the link between the virulent phenotype and
294 genotype to better understand the mechanisms of infection of this important human
295 pathogen.
296 There are some limitations to be considered in this study. First, the number of isolates was
297 small, however three different *A. fumigatus* population sources (clinical, environmental and
298 experimental) were included. Nevertheless, we encourage our findings to be confirmed in a
299 larger population to fully confirm the observation that all members of this species are
300 potentially pathogenic. Second, we included 244 genes in our in-house database and we do
301 not rule out the possibility that other genes that have not yet been characterized/described
302 could also be considered as VRG.

303 In conclusion, we developed an in-house database with 244 VRG and found them all (except
304 *Afu5g12720)* in the whole-genome sequence of nine *A. fumigatus* isolates, five clinical, two
305 environmental and two experimental. This indicates, the strain-specific virulence genomic
306 profile cannot be explained by the source of isolation when looking at differences in virulence
307 related gene content or sequence variations. Understanding under what circumstances VRGs
308 are expressed and utilized may ultimately contribute to explain how they regulate their
309 virulence. Moreover, a broad genomic variability and the convenient location of transposable
310 elements that are acknowledged to be shaping the genome, evidences a very efficient capacity
311 of adaptation and challenges the development of specific diagnostic tools and effective
312 treatments.
313
314 **Methods**
315 ***A. fumigatus* isolates and background**. *A. fumigatus* samples evaluated in this study are
316 summarized in Table 1. Four clinical isolates were included: three isolates (P1MS, P1MR and
317 P2CS) obtained at the University Medical Center Groningen (UMCG), Groningen, the
318 Netherlands, and the strain B5233, kindly provided by the Institute for Disease Control &
319 Prevention of the Academy of Military Medical Sciences, Beijing, China. B5233 is a clinical
320 isolate that demonstrated a high virulence in murine infection studies, and it has been used as
321 an experimental strain in *A. fumigatus* pathogenicity studies (35,36). The four isolates were
322 initially identified as *A. fumigatus* by microscopic morphological description and sequencing
323 of the internal transcribed spacer (ITS) region using Sanger sequencing.
324 P1MS and P1MR were originally isolated from the sputum of the same patient at different
325 time points during a complicated Influenza A H1N1 virus infection, and were regarded as
326 mixed infection isolates (Figure 1). This patient had no relevant underlying disease, was
327 diagnosed with Influenza A H1N1 virus and was admitted to the UMCG. Two days after
328 admission, a positive sputum culture of *A. fumigatus* prompted the initiation of treatment with
329 voriconazole. Later, at day five after admission, the patient developed IA and passed away
330 sixteen days after the diagnosis of the fungal infection. Throughout the course of the IA
331 infection (21 days), a total of seven *A. fumigatus* isolates were recovered, where the first five
332 isolates were susceptible to azole treatment and the last two were resistant. We selected the
333 first susceptible and the first resistant isolates to determine their genetic relatedness.
334 The remaining isolate, P2CS, was recovered from an individual diagnosed with HIV and COPD.
335 The *A. fumigatus* was cultured during a COPD exacerbation event. Chronic pulmonary

336 aspergillosis was discarded after a chest imaging study without the radiological
337 characteristics of pulmonary aspergillosis. Since no indicative symptoms of aspergillosis were
338 identified, the patient was regarded as colonized by this strain. The patient is still under
339 treatment with antiviral therapy ODEFSEY (emtricitabine/tenofovir alafenamide/rilpitvirine)
340 and treatment for COPD with fluticason, cotrimoxazol, formeterol and ipratropium.
341 In addition, the raw sequencing data of five unrelated Dutch and English isolates with an
342 environmental or clinical origin (22), were downloaded from the European Nucleotide
343 Archive (ENA) and included in the study (Table 1).
344 **Antifungal susceptibility testing** To determine the *in vitro* susceptibility of the clinical
345 isolates to triazole antifungal drugs the agar-based gradient technique for quantitative
346 antifungal susceptibility E-test (AB BIODISK, Solna, Sweden) was used for isolates B5233 and
347 P1MS, the agar-based method VIPcheck™ test (Nijmegen, The Netherlands) was used for
348 isolate P2CS and the susceptibility of P1MR was determined with the in vitro EUCAST broth
349 microdilution reference method (37).
350 **DNA isolation** Isolates were grown on Potato Dextrose Agar for 7 days at 35°C. DNA
351 extraction was performed with the DNeasy UltraClean Microbioal Kit (Qiagen, Hilden,
352 Germany) with some modifications to the initial steps of the manufacturer's protocol. The
353 initial fungal starting material was obtained using a pre-wetted sterile swab rubbed against
354 the sporulating colony, that was dissolved in 700 µl sterile saline solution. The suspension
355 was centrifuged at 10,000 rcf for 4 min. The supernatant was discarded, and the pellet was
356 resuspended in 300 µl of Power Bead solution. This solution was used to resuspend a second
357 pellet containing the same sample. The final concentrated solution was transferred to a
358 microtube containing Pathogen lysis tube L beads (Qiagen, Hilden, Germany), 50 µl of Solution
359 SL and 200 µl of sterile saline solution to homogenize. Disruption was carried out in a Tissue
360 Homogenizer Precellys 24 (Bertin, Montigny-le-Bretonneux, France), set to 3 times 30
361 seconds at 5,000 rpm separated by 30 seconds. The disruption preps were heated to 65°C as
362 suggested in the Troubleshooting Guide of the protocol to increase the final DNA yield
363 **Library preparation and whole genome sequencing.** The whole procedure was performed
364 according to the manufacturer's protocol (Illumina, California, United States of America). One
365 ng of fungal gDNA per specimen was used as input DNA for library preparation with
366 NexteraXT DNA Library Prep Kit. Library quality was determined by measuring fragment size
367 on a 2200 TapeStation System with D5000 Screen tape (Agilent Technologies) and quantified
368 with Qubit 2.0 Fluorometer using Qubit dsDNA HS Assay Kit (Life Technologies, ThermoFisher

369 Scientific, Waltham, Massachusetts, EUA). Before sequencing NexteraXT libraries were
370 denatured and diluted to the required (equi)molarity for Illumina platform and two pools
371 were made each containing two libraries. Whole-genome sequencing was performed in two
372 separate runs using the MiSeq Reagent Kit v2 500-cycles Paired-End on a MiSeq Sequencer
373 (Illumina). The raw reads generated in this study have been submitted to the European
374 Nucleotide Archive under project accession number PRJEB28819.
375 **Quality control and *de-novo* assembly.** The raw sequencing reads were quality trimmed
376 using the CLC Genomics Workbench software version v10.1.1 using default settings except for
377 the following modifications: "trim using quality scores was set to 0.01". Assembly quality data
378 of the nine *A. fumigatus* isolates is shown in Table S1. *De-novo* assembly produced acceptable
379 results that surpassed a >100 coverage with >90% of reads used.
380 **Identification of virulence related genes.** A database with genes associated with virulence
381 was generated based on Abad *et al* review (38). In addition, these genes were validated using
382 the online gene database AspGD (http://www.aspgd.org/). To increase the number of genes
383 and update the database, we added secondary metabolite (SM) genes from Biosynthetic Gene
384 Clusters (BGCs) 3, 5, 6, 14, 15 and 25, that showed a differential gene expression in murine
385 infection studies reported by Bignell *et al* (18). A list of allergens recently revised was also
386 included (39). A database with a total of 244 genes was created with genes categorized into
387 seven big groups according to their site and the process they are involved in, as follows:
388 thermotolerance, resistance to immune responses, cell wall, toxins and secondary
389 metabolites, allergens, nutrient uptake and signaling and regulation. (Table S2) The *de-novo*
390 assemblies of our isolates were screened with ABRicate v0.3 software tool
391 (https://github.com/tseemann/abricate) to detect the presence or absence of VRGs included
392 in the database. The thresholds were set to >90% coverage and >90% identity to determine
393 the presence of a virulence gene.
394 **TRESP genotyping.** This method is based on hypervariable Tandem Repeats located within
395 Exons of Surface Protein coding genes (TRESP) encoding cell wall or plasma membrane
396 proteins (20). The allele sequence repeats of three TRESP targets is combined to assign a
397 specific genotype: an MP-2 antigenic galactomannan protein (MP2), a hypothetical protein
398 with a CFEM domain (CFEM) and a cell surface protein A (CSP). The allele repeats of these
399 previously described proteins were used to Create a Task Template by Allele Libraries in
400 SeqSphere+ software v5.1.0 (Ridom GmbH, Münster, Germany) with import option: use as
401 reference sequence "best matching allele", which enabled a dynamic reference sequence. The

402 assembled genomes were imported into SeqSphere+ and the specific target repetitive
403 sequences of each protein were analyzed for each UMCG isolate using the Seqsphere+ "find in
404 sequence" tool to identify the specific genotype.
405 **Comparative genomics.** Genome assemblies of novel isolates were aligned using blast+ v2.6
406 (40) and reads were mapped with bowtie2 v2.2.5 (41) to the eight reference chromosomal
407 genomes of Af293 (NC_007194 -NC_007201). For each contig, local alignment coordinates
408 were extended to their whole length using the highest bitscore with an in-house script. Mean
409 coverage were calculated every 5 Kb using bedtools v2.17 (42). VRGs location were
410 determined by local alignment and GC percentage were calculated every 100 bp with
411 https://github.com/DamienFr/GC-content-in-sliding-window-script. Coding sequences, their
412 location and frame where extracted from the reference sequence genbank files. All gathered
413 information was represented in a circular image using circos v0.69-3 (43).
414 **Identification of variants.** The variant analysis was performed for the four UMCG isolates
415 and two Dutch environmental isolates 08-19-02-30 and 08-19-02-46. Variants were called
416 against the reference genome *A. fumigatus* Af293 (release 37, FungiDB) using the web-based
417 platform EuPathDB Galaxy Site(https://eupathdb.globusgenomics.org/) (44). The raw reads
418 were quality controlled with FastQC (version 0.11.3, Brabraham institute), trimmed with
419 Sickle (Galaxy version 070113) for quality and length thresholds of 20, aligned to reference
420 with Bowtie2 (Align version 2.1.0 64) (45) using the "very sensitive" alignment preset option,
421 BAM file sorted with SAMtools and variant calling performed with Freebayes (v0.9.21-19-
422 gc003c1e) and SAMtools (46). The resulting variants were annotated using SnpEff to predict
423 the impact of a variant on the effect of a gene, by classifying them in different categories: high,
424 moderate, low and modifier (47). (http://snpeff.sourceforge.net/SnpEff_manual.html)High
425 impact variants are predicted to have a disruptive effect in the protein (e.g. frameshift
426 variants, inversion), moderate impact variants could change the protein effectiveness (e.g.
427 missense variant, in frame deletion), low impact variants are not expected to have a big
428 impact in the protein function (e.g. synonymous variant) and finally, modifier variants are
429 non-coding changes where predictions are difficult or there is no evidence of impact (e.g. exon
430 variant, downstream gene variant). SnpSift was used to extract the variants with moderate
431 and high impact by filtering the resulting variant call format (VCF) files from SnpEeff.
432 In addition, identification of SNPs present in VRGs of our isolates, was performed using CLC
433 Genomics Workbench software version v11.0.1. For this approach, trimmed reads of each
434 genome were mapped to a concatenated sequence consisted of 244 VRG genes described in

435 Table S2. SNPs were called with a minimum read coverage of 10 and with a minimum
436 frequency of 90%. The used virulence gene sequences to create the concatenated sequence
437 belonged to the reference *A. fumigatus* Af293.
438 Snippy v. 4.3.5 (https://github.com/tseemann/snippy) was used to determine the number of
439 variants between isolate P1MS and P1MR. The trimmed reads of isolate P1MR were aligned to
440 the assembly of P1MS for variant calling. In this case, P1MS draft genome assembly, used as
441 reference, is not annotated and therefore, a functional prediction of the determined variants
442 was not possible and only a quantitative analysis is presented.
443

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455

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457 J.W.A.R consults for IDbyDNA. All other authors declare no conflicts of interest. IDbyDNA did
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460

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TABLE 1. Characteristics of *A. fumigatus* isolates investigated in this study.

Isolate	Country	Source	Resistance	Resistance mutation	Reference
B5233	The United States	Clinical/Experimental	Susceptible	-	(35,36)
P1MR	The Netherlands	Clinical (UMCG)	Resistant	TR34/L98H	This study
P1MS	The Netherlands	Clinical (UMCG)	Susceptible	-	This study
P2CS	The Netherlands	Clinical (UMCG)	Susceptible	-	This study
Af293 reference	Unknown	Clinical/Experimental	Susceptible	-	(22)
12-7505054	UK	Clinical	Susceptible	-	(22)
08-12-12-13	The Netherlands	Clinical	Resistant	TR34/L98H	(22)
08-19-02-30	The Netherlands	Environmental	Susceptible	-	(22)
08-19-02-46	The Netherlands	Environmental	Resistant	TR34/L98H	(22)

634

TABLE 2. TRESP genotype based on repetitive sequences in the exons of surface proteins CSP, MP2 and CFEM.

Sample	Allele CSP	Allele MP2	Allele CFEM	TRESP genotype
B5233	t02	m1.2	c09	t02m1.1c09
P1MR	t11	m1.1	c09	t11m1.1c09
P1MS	t03	m1.1	c08A	t03m1.1c08A
P2CS	t02	m1.1	c19	t02m1.1c19

635

TABLE 3. Variant analysis of the novel *A. fumigatus* isolates against reference *A. fumigatus* Af293.

Isolates	SnpEff Total number of variants	SnpSift Filter		SNPs present in the virulence genes (CLC Genomics Workbench)
		MODERATE	HIGH	
B5233	68,352	12,085	884	1122
P1MS	48,590	10,109	752	1107

P1MR	56,362	11,718	870	1015
P2CS	56,422	12,085	884	1158

TABLE 4. Examples of shared and unique moderate and high impact variants in genes associated to thermotolerance, resistance to the immune response, toxins and secondary metabolites, allergens and nutrient uptake.

Gene	Conserved in all	Per strain					
		B5233	P1MR	P1MS	P2CS	08-19-02-30	08-19-02-46
thtA	c.3698T>C c.3458T>C	c.3277C>T	-	c.2828C>T c.2188C>T c.1010G>T	c.3277C>T c.-598G>A	c.272C>A c.2128C>T HIGH stop gained p.Arg710*	-
pmt1	-	-	c.442G>A	-	-	-	-
rodB	-	-	-	-	-	-	-
cat1	-	-	-	-	-	-	-
catA	-	-	c.1385G>A	c.1385G>A c.982G>A	-	-	-
afpmt2	-	-	-	-	-	-	-
laeA	-	-	-	c.189G>A HIGH stop codon gained p.Trp63* c.400C>T	-	-	-
gliZ	-	c.1177C>G c.425_427delCAA disruptive_in frame_ deletion p.Thr142del	c.79A>G c.99_101dupTGC c.388A>G c.405_406insACAACAACAACA c.406_409delGCAGinsACAA	c.388A>G	c.388A>G	c.388A>G	c.1177C>G

			c.464T>G c.612C>T c.718T>G c.1087T>C	c.464T>G c.1087T>C c.397_409delGCAGCAGCAGCAGinsACAACAACAACAAAAACAA missense_variant&disruptive_inframe_insertion	c.464T>G c.1087T>C c.99_101dupTGC disruptive_inframe_insertion c.411_412insGCAACAACA c.412A>G	c.464T>G c.397_409delGCAGCAGCAGCAGinsACAACAACAACAAAAAA missense_variant&disruptive_inframe_insertion c.1338_1340delCTC disruptive_inframe_deletion c.1435A>G	c.412A>G c.406_411dupGCAGCA c.415A>G c.1404A>C
msdS	c.295T>C HIGH stop_lost p.Ter99Glnext*?	c.328C>G c.208T>A		c.328C>G c.208T>A	c.328C>G c.208T>A		c.328C>G c.208T>A c.1043C>T
sidC	c.3391A>G c.9598G>A c.9727T>C c.11935T>G c.14222G>A	c.577A>G c.1569C>G c.2311G>A c.3820A>G c.7174A>G c.11326A>G	c.577A>G c.1569C>G c.2311G>A c.3820A>G c.7174A>G c.3401C>T				c.577A>G c.1569C>G c.2311G>A c.3820A>G c.7174A>G

			c.13019A>T	c.751C>G c.13019A>T	c.878C>T c.11341C>T c.11935T>G	c.1781T>C c.4771C>A c.5798T>C	c.9769C>A c.13067A>C

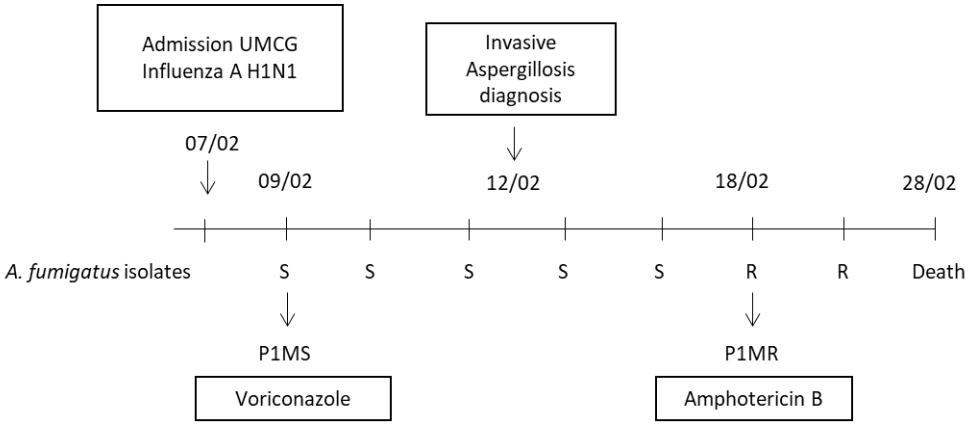
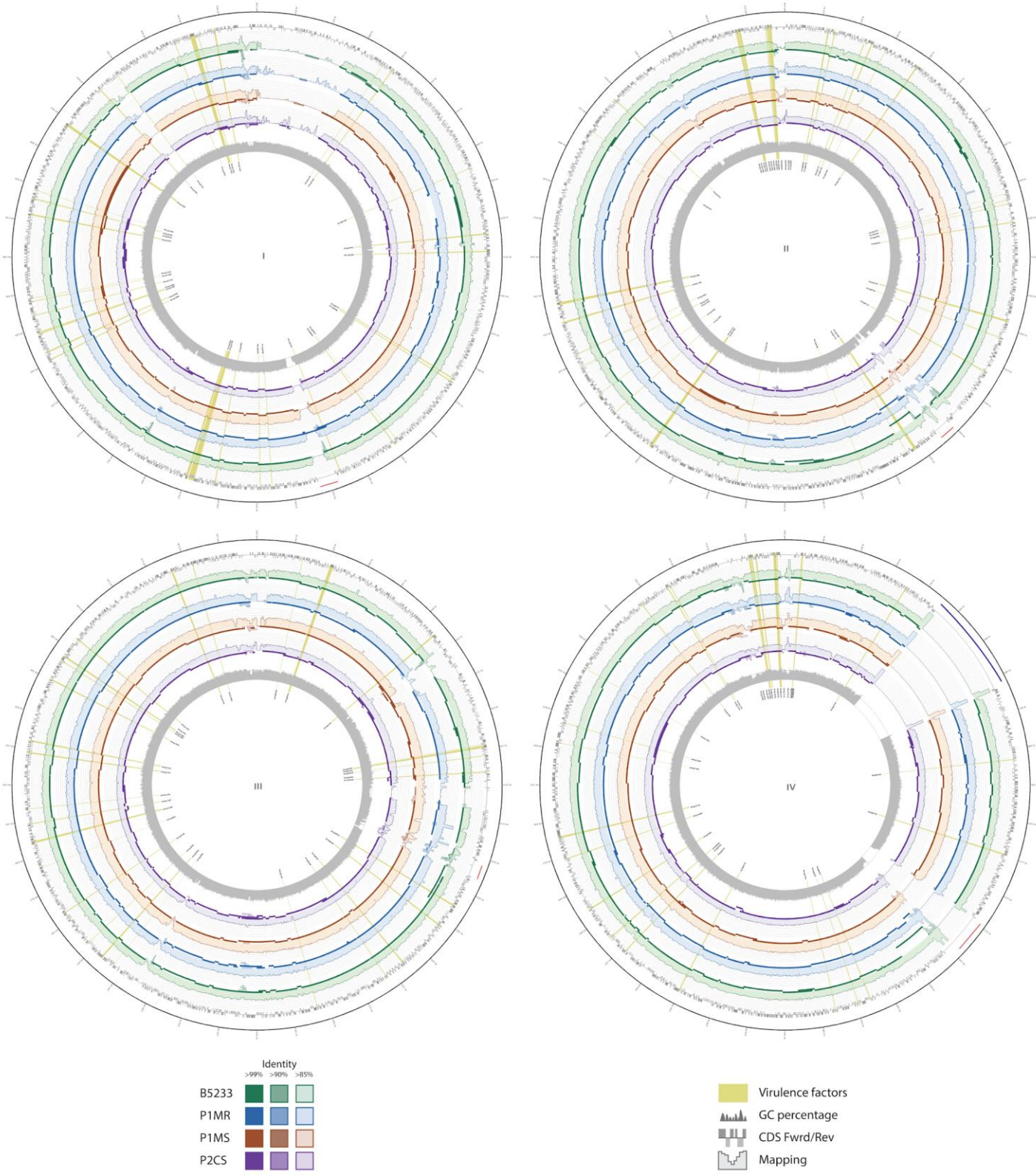


FIG 1. Time line of the influenza A H1N1 patient staying in the hospital and the course of infection. A total of seven *A. fumigatus* isolates were obtained from sputum samples, five susceptible (S) and two resistant (R) ones. The patient remained in the hospital for a period of 21 days until the time of death. Isolates P1MS and P1MR included in this study are indicated in the figure.


I
II
III
IV
Identity
>99% >90% >85%
B5233
P1MR
P1MS
P2CS
Virulence factors
GC percentage
CDS Fwrd/Rev
Mapping

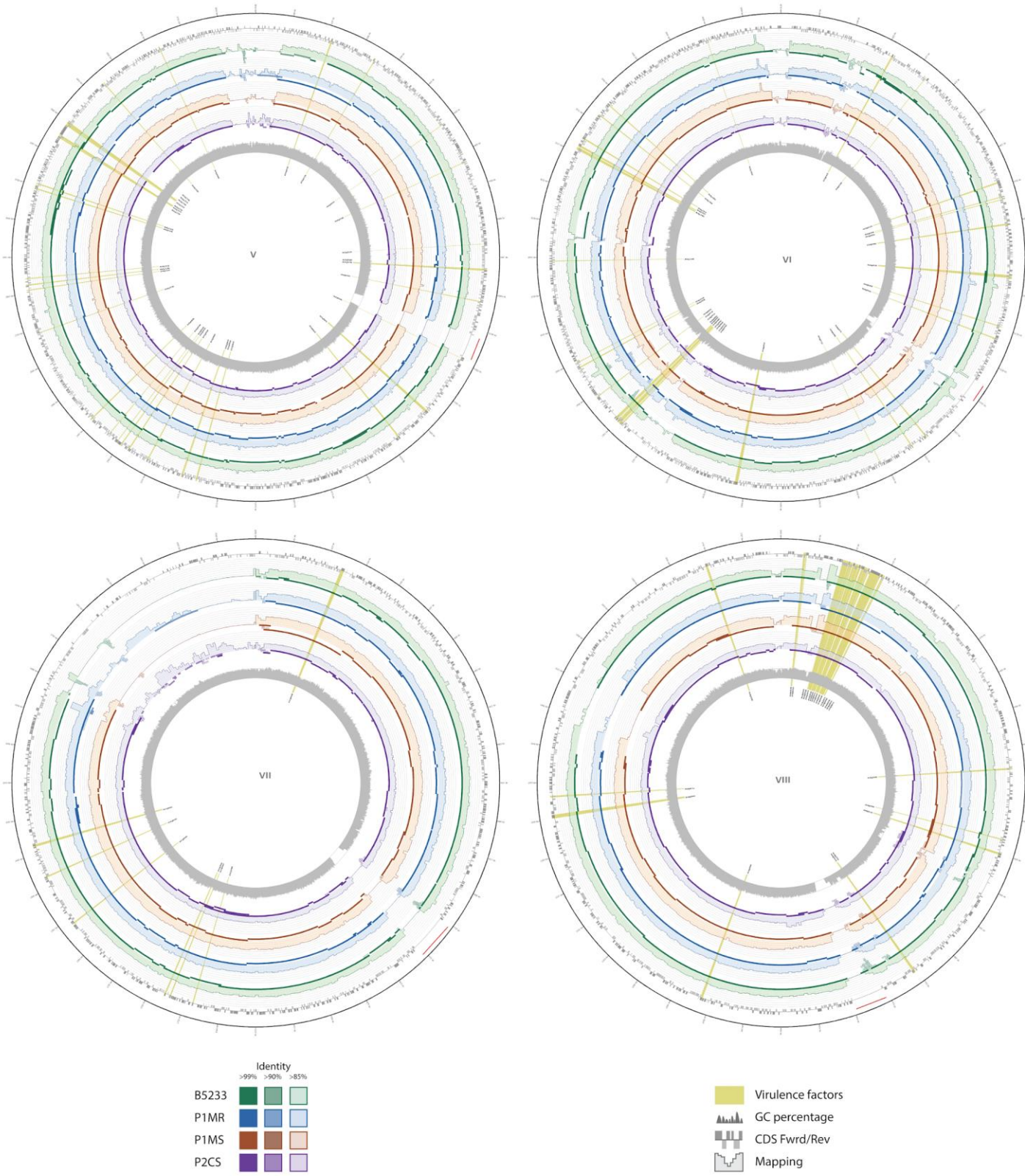


FIG 2. Graphical representation of assemblies and reads of B5233 (green), P1MR (blue), P1MS (orange) and P2CS (purple) isolates aligned to Af293 reference chromosomes (I, II, II, IV, V, VI, VII, VIII). Outer track indicates all CDS in forward (dark grey) or reverse (light grey) strand. Two different tracks are

represented per isolate: one corresponding the mapping coverage and another one corresponding to contig alignment (minimum ID 85%). The complete contigs are represented with transparency in accordance to the local alignment identity. Genes related to virulence are highlighted in yellow with its names in the innermost track. GC% is represented every 100 bp. Red lines indicate putative centromeres and the dark blue line (chromosome 4) represents ribosomal DNA.