Original Research

Heterogeneity in susceptibility to hydroxychloroquine of SARS-CoV-2 isolates

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1. Abstract

Background: Despite the fact that the clinical efficacy of hydroxychloroquine is still controversial, it has been demonstrated *in vitro* to control SARS-CoV-2 multiplication on Vero E6 cells. In this study, we tested the possibility that some patients with prolonged virus excretion could be infected by less susceptible strains. **Method**: Using a high-content screening method, we screened 30 different selected isolates of SARS-CoV-2 from different patients who received azithromycin ± hydroxychloroquine. We focused on patients with viral persistence, i.e., positive virus detection in a nasopharyngeal sample ≥10 days, and who were tested during two French epidemic waves, late winter-spring of 2020 and the summer of 2020. Dose-response curves in single-molecule assays with hydroxychloroquine were created for isolates with suspected reduced susceptibility. Genome clustering was performed for all isolates. **Results**: Of 30 tested strains, three were detected as replicating in the presence of azithromycin + hydroxychloroquine, each at 5 μM. The dose-response model showed a decrease in susceptibility of these three strains to hydroxychloroquine. Whole genome sequencing revealed that these three strains are all from the second epidemic wave and two cluster with isolates from Africa. **Conclusions**: Reduced susceptibility to hydroxychloroquine was not associated with viral persistence in naso-pharyngeal samples.

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Rather, it was associated with occurring during the second epidemic wave, which began in the summer and with strains clustering with those with a common genotype in Africa, where hydroxychloroquine was the most widely used.

2. Introduction

In December 2019, a novel coronavirus named SARS-CoV-2 emerged in Wuhan, China, in the province of Hubei [1–3]. SARS-CoV-2 spread rapidly around the world and the number of cases and deaths has increased rapidly (https://ourworldindata.org/coronavirus-data). Since then, finding effective treatments and vaccines has remained a major global challenge. Several drugs with an antiviral effect have been tested *in vitro* and *in vivo*, with drug repurposing being one of the strategies applied. Many drugs have shown an inhibition *in vitro* such as certain antimalarial drugs (chloroquine, hydroxychloroquine), ivermectin, macrolides (azithromycin, spiramycin), several protease inhibitors and some RdRp (RNA-dependent RNA polymerase) inhibitors such as remdesivir and sofosbuvir [4–18]. The combination of azithromycin and hydroxychloroquine showed synergistic effects *in vitro* [19] at concentrations of 5 μM for each drug and the combination was widely used in our institute to treat infected patients, as well as in several countries, particularly in Africa (Zambia, Uganda, Egypt, Algeria, Morocco, Tunisia, Senegal, Cameroon) [20, 21]. However, the use and effectiveness of this treatment remains highly controversial [22]. In the literature, and in our experience, some patients, especially immunocompromised patients or those with other comorbidities (such as hypertension and diabetes) have presented a positive viral load with a late clearance [23–26]. In our institute, we were able to isolate at least one SARS-CoV-2 strain from several patients who had been defined as having a "persistent" infection, with a positive RT-PCR test more than 10 days after admission, despite the fact that they had received a combination of azithromycin and hydroxychloroquine [27]. In those cases, the question of susceptibility to the antiviral drugs of the responsible strain was raised. Indeed, most *in vitro* studies evaluating susceptibility to antiviral drugs have used a unique strain or clone of SARS-CoV-2, and have considered that this clone is representative of all strains, despite the fact that the variability of antiviral activities on an enlarged panel of strains is unknown. In this study, we decided to use an automated model of Vero E6 to screen a single combination of azithromycin and hydroxychloroquine on multiple strains isolated from patients with persistent and non-persistent infections and who were randomly chosen to detect reduced susceptibility to this combination [28]. After this preliminary screening, a dose-response study to hydroxychloroquine was performed on suspect isolates and controls.

3. Materials and methods

3.1 Ethical concerns and sample collection

Nasopharyngeal samples were collected at the IHU Méditerranée Infection as part of COVID-19 diagnosis and patient follow-up. The study was approved by the ethics committee of the University Hospital Institute Méditerranée Infection (No.: 2020–029). With regards to both the French and the local situations, we defined two periods of time during the pandemic: the first consisting in the arrival of the virus and the lockdown between February and May 2020, and the second during the summer of 2020. Patients 6 to 15 were selected according to the persistence of their infection (defined by two positive PCR tests ten days apart). As stated above, all these patients were from the first wave of pandemic, between late February and May 2020 in our area. As controls we analysed a group of randomly chosen non-persistent control patients, patient 1 to patient 5 for the first wave of pandemic, and patient 16 to patient 20 for the second wave of pandemic, July–August 2020 in our area. Information on the sample collection, name of the strains, and treatments are summarised in Table 1. All patients received azithromycin that was, in most cases, combined with hydroxychloroquine [27]. For persistent patients, when it was possible, we evaluated the susceptibility of two strains, the first isolated upon admission and the second isolated during evolution under treatment. The IHUMI-3 isolate was among the first strains isolated in the laboratory and used as a control, as in our previous experiments [19]. Viral isolation was performed following the procedure described [29]. After isolation, the viruses were harvested and frozen at –80 °C. TCID50 (Tissue Culture Infectious Dose 50%) was performed for each strain and MOI (Multiplicity of Infection) for inoculation was adjusted according to the RT-PCR values in order to inoculate the same virus concentration for each virus. Before inoculations for antiviral assays, the viral stock was diluted in M4 medium.

3.2 Screening for reduced susceptibility and dose-response to hydroxychloroquine of selected isolates

All 30 strains from 20 patients (Table 1) and the IHUMI-3 control strain were screened using the high-content screening procedure for the combination of hydroxychloroquine and azithromycin (Sigma-Aldrich) at 5 μM each to evaluate the possible reduction of susceptibility. First, 200 μL of 5.105 cells/mL of Vero-E6 were incubated overnight at 37 °C with 5% CO_2 in 96-well plates. Supernatant was removed four hours before infection with SARS-CoV-2 and drug dilutions were incubated in the M4 medium four hours before. The viral infection of each strain was achieved with a MOI 0.001 (50 μL per well) except in negative controls. Imaging and cell analyses was performed by high-content-screening using the CX7 automated cell-

Table 1. Strains and patient information.

Patientnumber	Strain	Month of sampling	Persistence	Day of the latest isolate after onset	RT-PCR Ct values	Treatment	Clade	Access number
Patient 1	IHUMI-11	March 2020	No	NA	29	AZT, HCQ	20A/15324T	EPI_ISL_568957
Patient 2	IHUMI-15	March 2020	No	NA	23	HCQ	20B	EPI_ISL_568913
Patient 3	IHUMI-240	March 2020	No	NA	22	AZT, HCQ	20C-5	EPI_ISL_569338
Patient 4	IHUMI-243	March 2020	No	NA	29	AZT, HCQ	20A/15324T	EPI_ISL_569338
Patient 5	IHUMI-597	March 2020	No	NA	20	/	20A/25563T	https://doi.org/10.35081/8ytr-4812
Patient 6	IHUMI-215 IHUMI-611	March 2020	Yes	8	23 32,2	AZT, HCQ	20A/25563T-1B 20A/25563T-1B	EPI_ISL_2286971
Patient 7	IHUMI-364 IHUMI-599	March 2020	Yes	4	21 29,1	AZT, HCQ	20A/15324T	https://doi.org/10.35081/8ytr-4812
Patient 8	IHUMI-284 IHUMI-538	March 2020	Yes	4	30 20,5	AZT	20A/A0268G-2 20A/A0268G-2	https://doi.org/10.35081/8ytr-4812
Patient 9	IHUMI-713 IHUMI-800	March 2020	Yes	3	31 23,1	AZT, HCQ	20A/25563T-1 20A/25563T-1	https://doi.org/10.35081/8ytr-4812
Patient 10	IHUMI-684 IHUMI-743	March 2020	Yes	4	21,6 20,4	AZT, HCQ	20A/25563T 20A/25563T	https://doi.org/10.35081/8ytr-4812
Patient 11	IHUMI-598 IHUMI-801	March 2020	Yes	4	20,5 20,7	AZT, HCQ	20C-5 20C-5	EPI_ISL_569337
Patient 12	IHUMI-717 IHUMI-742	March 2020	Yes	2	21,2 19,1	AZT, HCQ	20B-1a 20B-1a	https://doi.org/10.35081/8ytr-4812
Patient 13	IHUMI-624 IHUMI-719	March 2020	Yes	2	16,1 17,7	AZT, HCQ	20A/25563T-1b	https://doi.org/10.35081/8ytr-4812
Patient 14	IHUMI-288 IHUMI-614	March 2020	Yes	5	23 26	AZT, HCQ	20C-4	https://doi.org/10.35081/8ytr-4812
Patient 15	IHUMI-880 IHUMI-990	April 2020	Yes	3	19 21,4	HCQ	20B 20B	EPI_ISL_568909
Patient 16	IHUMI-2122	July 2020	Unknown	NA	17,8	AZT	Marseille 1	EPI_ISL_569023
Patient 17	IHUMI-2123	July 2020	Yes	NA	17,7	AZT, HCQ	Marseille 1	EPI_ISL_569029
Patient 18	IHUMI-2137	August 2020	Yes	NA	14,7	AZT, HCQ	Marseille 5b	EPI_ISL_569119
Patient 19	IHUMI-2177	August 2020	No	NA	25,1	AZT	Marseille 1A	EPI_ISL_1745715
Patient 20	IHUMI-2178	August 2020	Unknown	NA	21,6	AZT, HCQ	Marseille 1A	EPI_ISL_569088

NA, not applicable because only one strain was obtained.

insight optical microscope (ThermoFisher Scientific, USA). The proof of concept used by Francis *et al.* [28] was developed to automatically detect infections in cells. Briefly, at time points H0 and 72 hours post-infection, wells were stained by NucBlue™ Live ReadyProbes™ reagent (Molecular Probes, Life Technologies, USA), at a final concentration of 2 ng/mL (5 μL per well directly from stock solution). Image acquisition and analyses were performed using the automated CellInsight™ CX7 High-Content Analysis Platform coupled with an automation system including an Orbitor™ RS Microplate mover and a Cytomat™ 2C-LIN (ThermoScientific) incubator. We evaluated the protective effect of A5H5 by comparison to the positive control without the addition of drugs and measured the difference in total cell count and % on infected cells according to the following formula: [total cell counts (A5H5-positive control)] * [% injured cells (A5H5-positive control)/10)]. As a consequence of this initial screening, three strains suspected to have possible reduced susceptibility to the combination (IHUMI-2123, IHUMI-2137 and IHUMI-2178) were first tested against hydroxychloroquine and azithromycin at 5 μM each, then in a serial dilution ranging from 25 μM to 0.39 μM of hydroxychloroquine to determine dose-response assays. In order to confirm that the effect was not a genotype-selection effect, we tested IHUMI-2122 and IHUMI-3 as control. A dose-effect curve was determined using a range of hydroxychloroquine doses (from 25 μM to 0.39 μM) at MOI of 0.001. Hydroxychloroquine dilutions were performed from a stock solution in M4 and then concentrations were adjusted. Each test was performed on at least six samples and repeated twice independently. The potential effect was monitored by RT-PCR after 48 hours of incubation under previously described conditions [30], with the exception of the polymerase being replaced by the SuperScript™ III Platinum™ SYBR with ROX (Sigma-Aldrich, catalogue number: 11736051). Relative viral quantification was performed compared to the positive control (viruses without drugs) by the 2-$\Delta\Delta$Ct (delta-delta Ct) method [31]. Statistical analyses were performed using GraphPad Prism v8.0.0 (GraphPad Software, La Jolla, California, USA).

3.3 Viral preparation and genomic sequencing, genomic assembly and bioinformatic analyses

Simultaneously with the antiviral assays, 500 μL of the viral supernatant obtained from co-culture was centrifuged through a UFC-filter (see previous section). Viral RNA was then extracted from 200 μL of the filtrate supernatant using the QIAcube kit. It was then reverse transcribed using SuperScript IV (ThermoFisher Scientific, Waltham, MA, USA) prior to cDNA second strand synthesis with Klenow Fragment DNA polymerase (New England Biolabs, Beverly, MA, USA). The next step concerned DNA purification which was performed using Agencourt AMPure XP beads (Beckman Coulter, Villepinte, France) and the sample was finally sequenced on Illumina technology with the Illumina Nextera XT Paired-end strategy on a MiSeq instrument (Illumina Inc., San Diego, CA, USA). The Wuhan-Hu-1 isolate genome served as a reference (consensus sequences GenBank Accession No. MN908947) and mapping was performed using CLC Genomics workbench v.7 (Fios Genomics, Edinburgh, UK). Sequences were compared to the GISAID database, and a phylogenetic tree was generated by using the nextstrain/ncov tool (https://github.com/nextstrain/ncov).

4. Results

4.1 High-content screening for reduced susceptibility detection

Of the 30 strains (plus the IHUMI-3 control strain) screened 72 hours after viral infection by SARS-CoV-2 on the high-content screening, with or without treatment by the combination of hydroxychloroquine and azithromycin both at 5 μM, IHUMI-2123, IHUMI-2137 and IHUMI-2178 had a low threshold obtained on the HCS software (1099, -1021 and -257 respectively), suggesting possible reduced susceptibility to A5H5 (Fig. 1A and **Supplementary Table 1**). It was difficult for us to decide on the status of IHUMI-2177, with a threshold obtained on the HCS software of 4192. This threshold is intermediate between the fully susceptible isolates and those with reduced sensitivity. This result was confirmed by SARS-CoV-2 replication analysis, even for IHUMI-2177 for which we observed reduction by drug combination, although this was not significant (Fig. 1B).

4.2 Dose-effect curves of hydroxychloroquine assays

Concerning the IHUMI-3 and IHUMI-2122 strains used as controls, we observed consistent viral inhibition compatible with the results previously observed in SARS-CoV-2 isolates. In contrast, concerning IHUMI-2123, IHUMI-2137 and IHUMI-2178, we observed a displacement of susceptibility to hydroxychloroquine (Fig. 2) confirming a specific pattern of reduced susceptibility for these isolates. For low concentrations of hydroxychloroquine ($<$3.125 μM), IHUMI-2177 behaves as strains IHUMI-2123, IHUMI-2137 and IHUMI-2178, suggesting reduced susceptibility to hydroxychloroquine. The lack of inhibition at low concentrations explains the large standard deviations. In contrast, for concentrations above 3.125 μM, strain IHUMI-2177 behaves like the control strains IHUMI-2122 and IHUMI-3, although the difference is not statistically significant. As suggested above, the status of IHUMI-2177 is difficult to assess, as it is intermediate between fully susceptible isolates and isolates with reduced susceptibility.

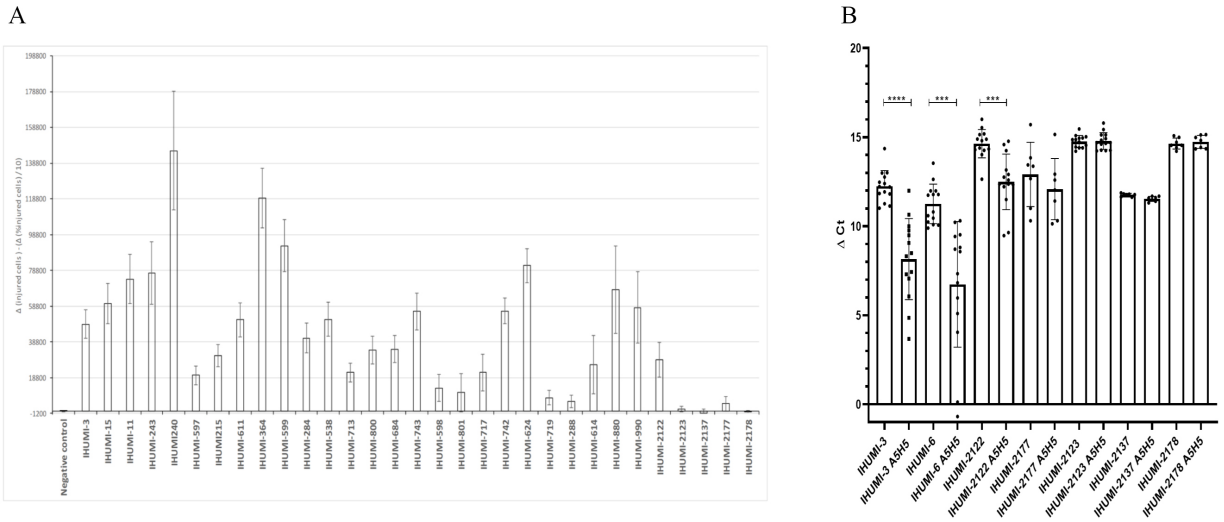

Fig. 1. Initial screening of the 31 selected SARS-CoV-2 isolates and a combination of hydroxychloroquine and azithromycin at 5 μM each. (A) Difference observed between cells treated or not treated calculated by high-content screening for each strain. (B) Effect of the association of hydroxychloroquine and azithromycin on SARS-CoV-2 replication on selected isolates. Delta Ct between 0 and 48 hours post-infection. The y-axis represents the variation of delta cycle-thresholds obtained by RT-PCR between H0 and H48 for each condition. Each point represents data obtained from one well. Median and interquartile range were indicated for each condition. *** represent significant results under $p < 0.0005$. Others are not significant compared to the control.

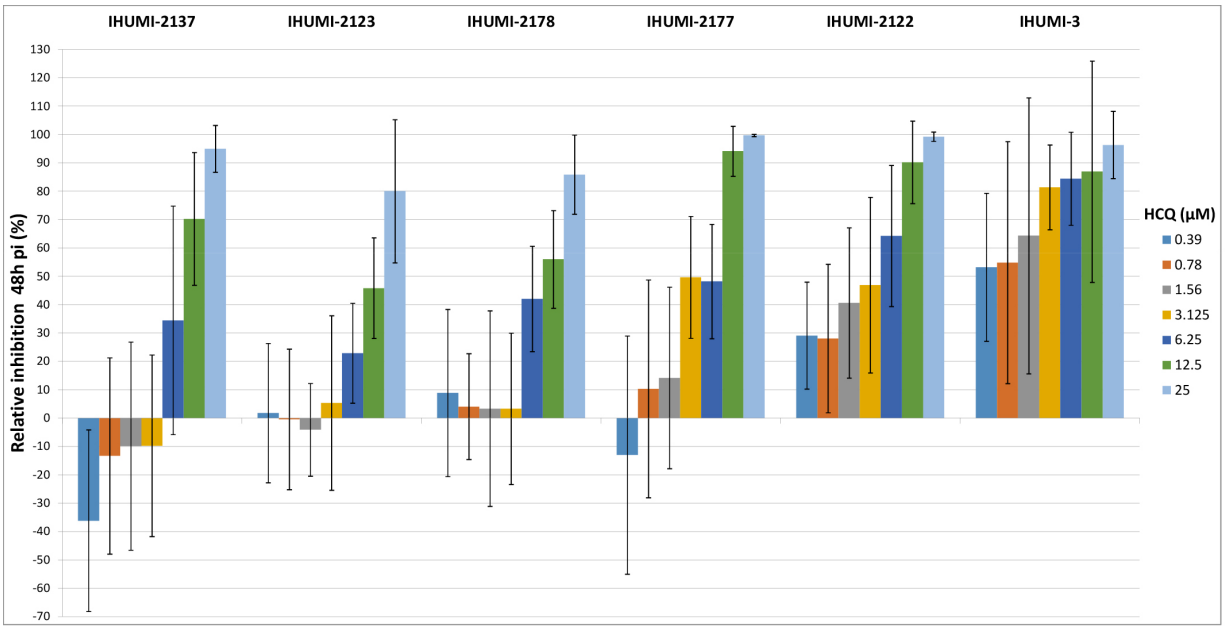

Fig. 2. Exploration of effect-dose of hydroxychloroquine. The range used from 25 μM to 0.39 μM tested on IHUMI-3, IHUMI-2122, IHUMI-2123, IHUMI-2137, IHUMI-2177 and IHUMI-2178 strains. Abbreviations: p.i., post-infection; HCQ, hydroxychloroquine; μM, micromolar.

4.3 Genome analysis

We first conduced a global genome-to-genome comparison on the couple of strains isolated in persistent patients and could not detect any modifications (Table 1). We also analysed 20 genomes to place them in a phylogenetic tree. Regarding the quality score on the next clade, all strains received a good quality score (**Supplementary File 1**). We were able to detect that all the strains from the second wave have ten or more amino-acid changes in their genome, compared to strains from the first wave. In contrast, all the strains of the first period had fewer than ten amino-acid mutations, with one exception (patient 12). All viruses in those studies had the D614G mutation in the spike, described elsewhere as potentially increasing the infectious effects [32]. The phylogenetic tree was reconstructed by integrating all IHUMI strains and evolutionary

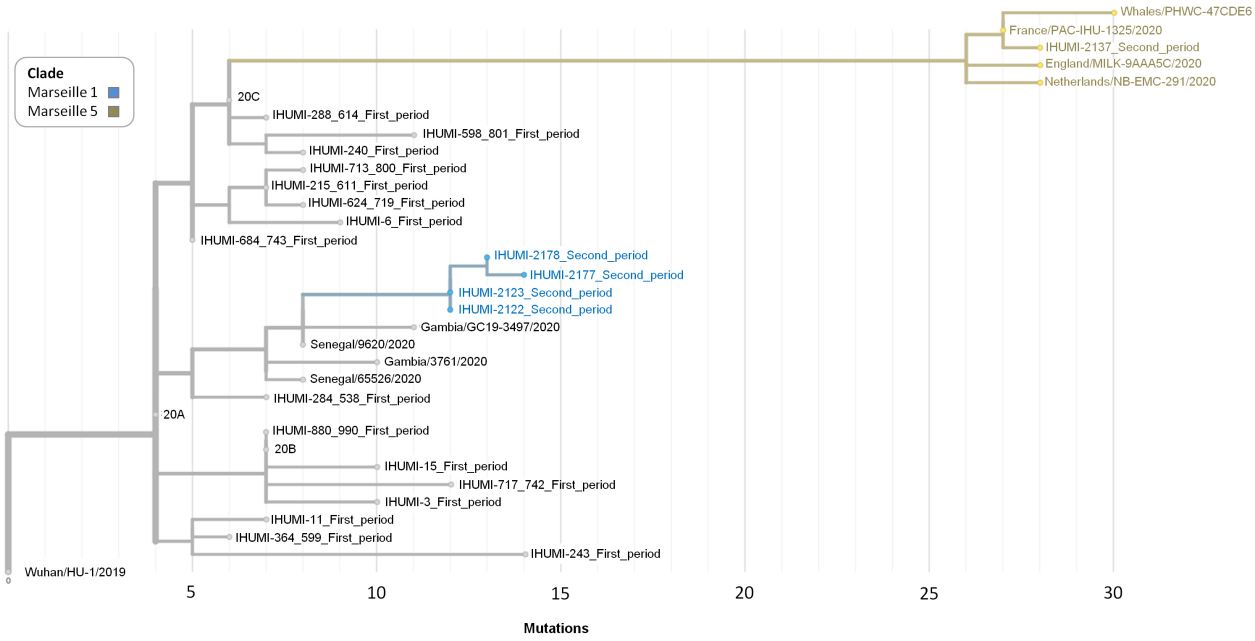

Fig. 3. Phylogenetic tree of whole genomes from IHUMI strains including closely related genomes available from GISAID. Mutation scales are compared to the Wuhan reference genome.

relationships were revealed (Fig. 3). All five strains from the second period belonged to separate clades, provisionally named Marseille 1 and Marseille 5 [33]. Specifically, the strains with reduced susceptibility to hydroxychloroquine were from Marseille clades 1 (IHUMI-2123 and IHUMI-2178) and 5 (IHUMI-2137). All strains belonging to the Marseille 1 clade were positioned close to genomes originating from Africa, specifically Senegal and Gambia. The phenotype with reduced hydroxychloroquine in Marseille 1 genotype was shared by the IHUMI-2123 and IHUMI-2178 isolates, possibly IHUMI-2177, but not by the IHUMI-2122 isolate. IHUMI-2137 grouped within the Marseille 5 clade. Meanwhile, the IHUMI-2123, IHUMI-2178 and possibly IHUMI-2177 isolates with a reduced susceptibility to hydroxychloroquine, did not present mutations as compared to the IHUMI-2122 isolate without reduced susceptibility.

5. Discussion

To the best of our knowledge, this is the first time that three SARS-CoV-2 strains have been shown to have a profile of reduced susceptibility to hydroxychloroquine *in vitro*. Susceptibility to azithromycin was not tested independently, as its effect alone *in vitro* is limited. In this study, the high-content screening technology, first applied to the high-throughput culture of giant viruses of amoeba then to SARS-CoV-2 [30, 34], was used for the first time to rapidly screen for the susceptibility to drugs of a large panel of viruses. Although other research will be needed to clearly confirm that the procedure can be sufficiently standardised to provide efficient large screening of strains, it has been shown to be efficient at detecting isolates with reduced susceptibility. However, although highly time-consuming and susceptible to many confounding factors, as presented below, when it comes to the fine determination of susceptibility, dose-effect determination using molecular biology remains necessary. Indeed, *in vitro* sensitivity assays carried out on the same virus can provide divergent results, reflecting great discrepancies due to several key determinants in the experiments. First of all, the cell lines used may harbour different permissivity levels, resulting in differences in viral titres, although those used in these assays needed to be permissive. For SARS-CoV-2, the entry step involves the ACE2 receptor and two independent host protease pathways, TMPRSS2 or the cathepsins B/L that activate the spike viral protein. The virus may not use these two pathways in the same manner, and the expression level of these receptors mediating virus entry are differentially expressed according to the cell lines [35]. For example, VeroE6 engineered to express greater amounts of TMPRSS2 has been used elsewhere, resulting in 100-fold higher titres of SARS-CoV-2 [36]. Inversely, viral titres provided by SARS-CoV-2 infected Calu-3 cells (continuous human lung epithelial cell line) are lower than in Vero cells [36]. It could make sense for the sensitivity assays to use the cells physiologically closest to those of the replication site *in vivo*. From this perspective, primary cells derived from organ explants were used for sensitivity assays and seemed to present a relevant approach. However, variable effects which are donor-dependent on the sensitivity for some tested drugs

should be expected due to differences in viral replication and gene expression [37]. Thus, this approach could be a "false" good idea and testing molecules in a coarse model such as in Vero E6 that has genetic defects in terms of interferon production could help provide evidence of such an effect. Secondly, the multiplicity of infection reported for the drug concentration is not standardised. It seems obvious that the higher the MOI, the lower the relative drug concentration, and the more likely the virus can replicate. This MOI is not even mentioned in some studies. Finally, the time of end point evaluation and the method used for assessing viral replication also varies according to the study from one hour to 120 hours [38, 39]. In addition, the assessment of viral replication by PCR or fluorescent assay or visual inspection to monitor cell viability may not have the same sensitivity. For example, when it comes to visual inspections, some permissive cells such as human intestinal epithelial Caco-2 do not produce cytopathic effects after SARS-CoV-2 infection and thus cannot be evaluated using such a method [40]. As a result, it is risky to draw conclusions on the basis of a single sensitivity test, especially when testing a virus with a high genomic variability.

One of the most interesting perspectives should also be to test multiple viral strains to check the concordance of the results. Currently, *in vitro* assays essentially use one or two SARS-CoV-2 strains. Our work suggests it is risky to draw conclusions on a single sensitivity test when testing a virus with a high genomic variability. Indeed we observed heterogeneity in the hydroxychloroquine antiviral activity screening of 30 strains and were able to detect three strains with a lower susceptibility profile. For isolates from patients during the first wave of the epidemic, persistence was clearly not associated with a lowered susceptibility profile to hydroxychloroquine *in vitro*. This confirmed the observation that persistence and severity are rather associated with host factors, as suggested by recent genetic research on COVID-19 severity-associated factors [41, 42] or immunocompromised status [43, 44]. Moreover, genomic analyses did not reveal any modification in these isolates that could explain persistence, neither in the sequence of the strain isolated upon admission nor in that of the strain isolated during the course of the disease. The less susceptible hydroxychloroquine strain, IHUMI-2123, which belongs to Marseille 1 genotype, was isolated in early summer 2020 at the beginning of the second wave, from a patient returning from Tunisia [33], a country in which hydroxychloroquine was massively used [21]. We evidenced a close phylogenetic proximity between all strains of the Marseille 1 clade (IHUMI-2123, IHUMI-2122, IHUMI-2178, and IHUMI-2177) with strains isolated in Senegal and Gambia, two countries which use hydroxychloroquine to treat patients with COVID-19 [45, 46]. We believe that it is possible that the widespread use of hydroxychloroquine in these countries led to strains being selected with reduced, which were later transmitted to the Marseille population. This observation is worthy of analysis on a statistically larger number of strains of this genotype. Paradoxically, the patients tested in Marseille hospitals during the early summer of 2020 and infected by isolates of this genotype presented milder infections and lower mortality than that observed during the first wave of the epidemic, despite the fact that the viral loads in their respiratory secretions were higher. This observation raises several questions that will be difficult to resolve, such as "does the lowered susceptibility to hydroxychloroquine reduce the severity of infection?" or "is it useful to use hydroxychloroquine in such cases or only in patients with severity markers or risk factors such as anticoagulant lupus, which is treated with hydroxychloroquine and the likely efficiency of which is therefore not due to an antiviral effect?" [47–49]. Finally, the hydroxychloroquine concentration to achieve 50% of viral inhibition was around 3.125 μM for the two strains with high hydroxychloroquine susceptibility and $>$12.5 μM for the three strains with reduced susceptibility, which require at least four times more hydroxychloroquine for the same effect (Fig. 2). Moreover, a 90% viral inhibition required around 12.5 μM for susceptible strains and $>$25 μM for less susceptible strains. However, these concentrations remain consistent with concentrations observed in human plasma and lungs. An oral intake of 400 mg of hydroxychloroquine led to a maximum blood concentration (Cmax) of 1.22 μM [50]. However, hydroxychloroquine accumulated 30 times more in the lungs than in the blood [51], allowing a potential efficiency of hydroxychloroquine even against strains with reduced susceptibility. An oral intake of 400 mg of hydroxychloroquine would still be effective *in vivo* in humans infected with the current strains with *in vitro* reduced susceptibility to hydroxychloroquine.

However, these genotypic and phenotypic variations could be frequent in the viral populations in the future and could apply to more drugs and need to be considered in the global repurposing strategy. Following the description by Korber *et al.* [32], we know that the spike population evolved between February and April 2020 and constituted a fast replacing situation by the G614. Recently, a major situation was reported in Denmark, where minks were infected with a strain presenting a few mutations, notably in the spike protein and associated with a selection pressure in a potential zoonotic transfer. Those aspects need to be carefully considered, in terms of testing and using antiviral compounds, but also in terms of epidemiology and vaccination strategy.

6. Conclusions

To summarise, we observed that the persistence of SARS-CoV-2 in some patients was unrelated to any decrease in the *in vitro* sensitivity of their strains to hydroxychloroquine. In contrast, we unexpectedly found, in some control strains, a decrease in sensitivity. This characteristic

is linked to particular genotypes observed during the second wave of the epidemic in our region and the emergence of the first variants, which were certainly imported from Africa. Finally, it should be recalled that these simple cell models on Vero cells, even if they certainly reflect specific characteristics of the strains tested, are not necessarily relevant for use in humans. *In vitro* work on other cells such as Calu-3 lung cells [52] or using animal models such as ferrets [53] show a clear divergence of the effect of the drugs tested compared to the Vero model.

7. Author contributions

Conceptualization—PC, AL and BLS; software—JD, SA and AL; Investigation—CB, MLB, JA, PJ and MG; formal analysis—CB, MLB, JA; writing—original draft preparation—CB, MLB, JA, MG; writing—review and editing—PC, BP, AL and BLS.

8. Ethics approval and consent to participate

The collection of strains was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethical committee of the University Hospital Institute Méditerranée Infection (No: 2020-029). Patient consent was waived due to the fact that diagnosis by culture was part of routine diagnosis procedure and patients' data anonymized.

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11. Conflict of interest

The authors declare no conflict of interest.

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