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Running title: Dynamics and genetic diversity of *H. influenzae*

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Abstract

Background: To investigate the genetic diversity of *Haemophilus influenzae* positive samples among French pilgrims attending the 2018 Hajj pilgrimage.

Method: After screening by qPCR, multilocus sequence typing was performed for all *H. influenzae*-positive samples. The following housekeeping genes were amplified and assigned: *adk, atpG, frdB, fucK, mdh, pgi* and *recA*.

Results: 121 pilgrims were included. *H. influenzae* was positive in 35.5% pre-Hajj samples, 12.4% at day five post-arrival, 15.7% at day 12 post-arrival, and 43.0% post-Hajj. Of the 129 positive swabs for *H. influenzae*, only one sample at D12 was negative for all seven genes amplified by standard PCR. The *adk, atpG, frdB, mdh, pgi, recA* and *fucK* genes were positive in 123, 107, 122, 70, 127, 118 and 69 samples, respectively. One sequence of *atpG* and two of *recA* genes were not possible to assign. None of the sequences of *fucK* gene was successfully obtained. Consequently, a complete sequence type characterisation was not possible. Of the 128 obtained strains, 111 had distinct patterns of alleles.

Conclusion: *H. influenzae* genotypes acquired were completely different from those present at pre-Hajj. We observed a great biodiversity and a lack of clonality of *H. influenzae* among French pilgrims during the 2018 Hajj. Further studies aiming at studying the genome of Hajj-acquired *H. influenzae* isolates are needed to define the clinical burden of *H. influenzae* infection during Hajj and to evaluate the potential interest of vaccination in Hajj pilgrims.

Key words: Hajj, pilgrims; *Haemophilus influenzae*; MLST; genetic diversity

Introduction

Every year, the Kingdom of Saudi Arabia (KSA) hosts more than two million people from about 180 countries during the Hajj pilgrimage. This number continues to increase, and the estimated number of annual attendees is expected to reach 4.5 million by 2030 [1]. Crowding and the extreme heat in Mecca, are known risk factors for the transmission of respiratory infections at the Hajj [2]. Infectious diseases accounted for 53% of diagnoses in outpatients consulting at the Indian Medical Mission in Mecca during the 2014–2016 Hajj, with respiratory tract infections (RTIs) and gastroenteritis being the most common [3]. The effectiveness of individual non-pharmaceutical preventive measures against infectious diseases is uncertain [3]. Despite several preventive measures [4], including hand hygiene, handkerchief use, face mask use, and vaccinations against influenza and pneumococcal infections in at-risk individuals being recommended, RTIs remain frequent among Hajj pilgrims. As an example, up to 90% of French pilgrims suffered an RTI during the 2012–2014 Hajj [5, 6]. Besides the widespread acquisition of respiratory viruses, bacteria are also frequently isolated among ill and asymptomatic pilgrims with *Haemophilus influenzae* being the most common [7-10]. Most cohort surveys among pilgrims used real-time PCR to detect the carriage of *H. influenzae*. Based on a culture method, Nik Zuraina showed that *H. influenzae* was the most predominant bacterium isolated (60%) from Malaysian pilgrims with RTIs during the Hajj [9]. In a 2018 study conducted among French Hajj pilgrims the carriage of *Staphylococcus aureus, Moraxella catarrhalis, Streptococcus pneumoniae and Klebsiella pneumoniae* increased following participation in the Hajj [11]. In contrast, *H. influenzae* carriage first decreased on D5 and D12 post-arrival by a factor of 2.5 and then increased in post-Hajj samples to reach carriage rates higher than that of pre-Hajj samples

[11]. The objective of this study was to investigate the genetic diversity of these *H. influenzae* positive samples among French pilgrims before, during and after the 2018 Hajj.

Methods

Participants and study design

This study included pilgrims travelling from Marseille, France, to Mecca, KSA during the 2018 Hajj. Participant were recruited at a single specialized travel agency and followed-up during the travel by two bilingual (French and Arabic) medical doctors. All pilgrims departed to KSA at the same date, were housed in the same accommodation and performed the rituals together during their stay. To evaluate the dynamic of *H. influenzae* during the Hajj, all pilgrims underwent four successive systematic nasopharyngeal swabs at different times: pre-travel, five to six days post arrival (D5 sample), 12 to 13 days post arrival (D12 sample) and just prior to leaving KSA (post-Hajj sample). The Hajj rituals took place from 19–24 August, corresponding to days 14 to 19 post-arrival (Figure 1).

Respiratory specimen and screening for *H. influenzae by real-time qPCR*

The sampling was done by the doctors accompanying the group, in a standardized way (3 cm in the nostril, 5 turns; post wall of the pharynx, 5 streaks). Obtained swabs were transferred to Sigma-Virocult® medium and kept at room temperature (20°C) during travel and then stored at -80°C until processing.

The DNA were extracted from the samples using the EZ1 Advanced XL (Qiagen, Hilden, German) with the Virus Mini Kit v2.0 (Qiagen) according to the manufacturer's

recommendations. All quantitative real-time PCR were performed using a C1000 Touch™ Thermal Cycle (Bio-Rad, Hercules, CA, USA).

Real-time PCR amplifications were carried out using LightCycler® 480 Probes Master kit (Roche diagnostics, France) according to the manufacturer's recommendations. The *SHD* gene of *H. influenzae* was amplified with internal DNA extraction controls TISS, as previously described [12]. Negative controls (PCR mix) and positive controls (DNA from *H. influenzae* strain) were included in each run. Positive results of DNA amplification were defined as those with a cycle threshold (CT) value ≤35.

Multilocus sequence typing (MLST) of *H. influenzae*

MLST was performed for all positively screened samples. The housekeeping genes *adk, atpG, frdB, fucK, mdh, pgi* and *recA* were amplified by standard PCR, as previously described [13], then sequenced. The sequences were compared to those existing in GenBank. Positive samples which presented at least one of seven genes using standard PCR were considered as being *H. influenzae* MLST positive samples. Purified PCR products were sequenced using specific primers and the BigDye Terminator® version 1.1 cycle sequencing ready reaction mix (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using an Applied Biosystems 3130 platform (ABI PRISM, PE Applied Biosystems, USA). The sequences obtained were edited and assembled using Chromas Pro 1.77 (Technelysium Pty Ltd, Australia), aligned with *H. influenzae* strains from GenBank. Allele numbers and sequence types (ST) were assigned using the *H. influenzae* MLST website (http://haemophilus.mlst.net).

Results

Characteristics of study participants and clinical symptoms

The characteristics of study participants and clinical symptoms have been detailed elsewhere [11]. To summarise, 121 pilgrims were included, with a sex ratio of 1:1.3 and a median age of 61 years (interquartile = 56-66 years, range = 26–83 years). A total of 113/121 (93.4%) pilgrims presented at least one respiratory symptom during their stay in the KSA. Antibiotic use for RTIs was reported by 58.7% pilgrims. The mean time between arrival in the KSA and the onset of respiratory symptoms was 8.7 ± 4.6 days (range = 1 – 21 days).

Identification of *H. influenzae* among French pilgrims during the Hajj

***H. influenzae* screening by real-time PCR**

Of the 484 swabs tested, 129 (26.7%) were positive [11]. Of the 121 pilgrims included, 75 (62.0%) at least one of their samples was positive for *H. influenzae* and all four samples of the remaining 46 were negative. The prevalence of *H. influenzae* carriage was 43/121 (35.5%) in samples obtained before leaving France, 15/121 (12.4%) at D5, 19/121 (15.7%) at D12 and 52/121 (43.0%) before leaving Saudi Arabia [11].

Diversity of encapsulated and non-capsulated *H. influenzae* using multi-locus sequence typing

Of the 129 swabs that were positive for *H. influenzae*, only one sample at D12 was negative for all seven genes amplified by standard PCR. Figure 2 shows the distribution of the 128 MLST positive samples among the 75 pilgrims who presented at least one positive sample. A total of 40/121 (33.0%) pilgrims presented only one of the four samples that was positive for at least one of the seven genes (14 at pre-Hajj, 1 at D5, 3 at D12 and 12 at post-Hajj samples). In addition, 21/121 (17.4%), 10/121 (8.3%) and 4/121 (3.3%) pilgrims had two, three and four MLST positive samples, respectively.

The *adk, atpG, frdB, mdh, pgi, recA* and *fucK* genes were positive in 123, 107, 122, 70, 127, 118 and 69 samples, respectively. All sequences of *adk, frdB, mdh* and *pgi* genes were obtained from GenBank or using the *H. influenzae* MLST website (GenBank accession numbers MN555328, MN607727–MN607848, MN617868–MN617990, MN617991–MN618060 and MN627489–MN627615). A total of 106/107 and 116/118 sequences of *atpG* and *recA* genes were assigned with success respectively (GenBank accession numbers MN607849 – MN607953 and MN627616 – MN627731). One sequence of *atpG* and two of *recA* genes were not possible to assign, using Chromas Pro 1.77. However, none of the sequences of the *fucK* gene was successfully obtained (Figure 3). Consequently, complete sequence type characterisation was not possible. Nevertheless, our results enabled us to differentiate between strains. The supplementary data shows the diversity of the six genes obtained from pilgrim strains. Of the 128 obtained strains, 111 had distinct patterns of alleles. In post-Hajj samples, five distinct strains were each found in two pilgrims and one was found in three pilgrims. Samples with similar strains were also found in one pre-Hajj and one post-Hajj samples in two distinct individuals and another strain was found in one D12 and one post-Hajj samples in two other pilgrims.

Discussion

In this study, we evaluated the dynamics and genetic diversity of *H. influenzae* carriage among French pilgrims during the 2018 Hajj season. Our main results are as follows: 1 - *H. influenzae* carriage was frequent among pilgrims before leaving France and increased following participation in the Hajj, following a transient decrease; 2 - *H. influenzae* genotypes acquired in the KSA were completely different from those present before leaving France and 3 - we

observed a great biodiversity and a lack of clonality of *H. influenzae* among pilgrims during the Hajj.

H. influenzae is a commensal organism of the human respiratory tract and is a common cause of upper and lower RTIs in adults [14]. Occasionally, it also causes life-threatening invasive diseases such as meningitis in adults [15]. This organism has been described as commensal in 75% of the healthy adult population [15] and typing of the *H. influenzae* isolates is essential in order to confirm its pathogenicity in pilgrims. Within the species, *H. influenzae* type b (Hib) is known to cause severe forms of infections [16]. Non-capsulated (non-typeable) *H. influenzae* is commonly carried in the pharynx and is one of the major causes of acute otitis media in children. It can cause diseases of the upper and lower respiratory tracts, including sinusitis and pneumonia [13]. However, previous studies conducted in some countries using the Hib conjugate vaccines reported that non-typeable and non-b serotypes caused most invasive diseases in adults over the age of 65 [17-19]. The World Health Organization recommends the epidemiological surveillance of *H. influenzae* to evaluate the current burden of its associated diseases and, consequently, to ascertain the practicality of Hib vaccine in the affected regions.

Pneumonia is a leading cause of hospitalisation during the annual Islamic pilgrimage. Memish conducted a study among pilgrims with severe community-acquired pneumonia who were admitted to 15 healthcare facilities in the cities of Makkah and Medina in Saudi Arabia [20]. Bacterial pathogens were detected in 84.6% of cases. *H. influenzae* and *S. pneumoniae* were the predominant bacteria, detected in 57.7% and 53.8% of patients, respectively. In a cohort study conducted among international pilgrims, Memish showed that the carriage of *H. influenzae* detected by qPCR was low. Only 2.3% of pilgrims were positive for this bacterium pre-Hajj and 11.7% post-Hajj [21]. Likewise, in a longitudinal survey conducted in 254,823 Iranian pilgrims

recruited at 1,352 Hajj caravans over a five-year period (2004-2009), *H. influenzae* was identified in only 9.1% of the 357 tested samples [22]. Among French pilgrims from Marseille, *H. influenzae* pre-Hajj prevalence varied according to the Hajj season: 50% in 2014, 0.9% in 2015, 2.8% in 2016 and 52.8% in 2017, while post-Hajj prevalence was consistently high with 67.8%, 45.5%, 41.0% and 53.5%, respectively [8, Gautret, unpublished data]. This suggests that *H. influenzae* carriage in Marseille may be subject to yearly variations. In contrast, a frequent acquisition of this bacterium at the Hajj is observed each year. In line with this result, Wilkinson conducted a prospective analysis in the United Kingdom, surveying 127 patients with chronic obstructive pulmonary disease (COPD) aged between 40 and 85 over a period of two years (from June 2011 to June 2012) for the occurrence of acute exacerbation and respiratory infections in COPD. Their results showed, for the first time, that changes in the yearly COPD exacerbation rate may be associated with variations in *H. influenzae* colonisation [23].

In our study, *H. influenzae* alleles varied according to the sampling time during Hajj. In pilgrims who were positive for *H. influenzae* in all four samples, *H. influenzae* genotypes were different in most samples, suggesting that pilgrims successively acquired different new *H. influenzae* genotypes during the pilgrimage. The reason for the initial clearance of *H. influenzae* carriage following arrival to the KSA may have resulted from the high rate of antibiotic consumption by pilgrims [24]. The large diversity of genotypes acquired during the stay in the KSA suggests that the source of infection was polyclonal and was likely to be external to the group of French pilgrims, with very little transmission between them.

In our study, none of the sequences of the *fucK* gene was successfully assigned. Other researchers have reported that certain *H. influenzae* isolates may lack the *fucK* gene, one of the seven genes used in the *H. influenzae* MLST scheme [13, 25-27]. The absence of this gene has

historically confounded attempts at *H. influenzae* speciation, such as the probable incorrect assignment of *fucK*-negative *H. influenzae* strains as *H. haemolyticus* [28]. These 'fuzzy' isolates should therefore be considered to be fucose-negative *H. influenzae*. It is unfortunate that the developers of the *H. influenza* MLST scheme inadvertently chose a variably present gene for genotyping purposes. Price confirmed the existence of fucose-negative *H. influenza* strains [29]. Redesign of the existing *H. influenzae* MLST scheme to incorporate a conserved seventh locus in *H. influenzae* and *H. haemolyticus* in place of the *fucK* gene may lead to greater uptake of this highly useful genotyping scheme around the world.

Our study has several limitations. First, we performed sequencing directly from samples, not on cultured isolates and we could not succeed in amplifying all seven genes in the MLST system. In addition, we could not eliminate the possibility that an individual sample could be positive for several genotypes of *H. influenzae*. The study was conducted among French pilgrims only and cannot be generalised to all pilgrims. Using qPCR to detect respiratory pathogens does not distinguish between dead and living micro-organisms. Nevertheless, our study is the first study on the dynamic and genetic diversity of *H. influenzae* in the Hajj context. Continuous monitoring of the molecular evolution in *H. influenzae* among Hajj pilgrims is necessary to explore how *H. influenzae* biofilm formation involves in the pathological process of infection and may help for clinical treatment. Moreover, constant vigilance and precaution such as a vaccine development is necessary to respond to new epidemiological trends of *H. influenzae* strains acquired by Hajj pilgrims. *H. influenzae* type b vaccination has been described by previous study that induces serum antibody production and reduces the nasopharyngeal carriage prevalence of this bacterium. Reduction in carriage also reduces transmission of *H. influenzae* between individuals [30]. Further studies aiming at studying the genome of Hajj-acquired *H. influenzae* isolates are

needed to better elucidate their ecological changes, to define the clinical burden of *H. influenzae* infection during Hajj and to evaluate the potential interest of vaccination in Hajj pilgrims. Currently, vaccination against invasive *H. influenzae* disease is not recommended for Hajj pilgrims [4].

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Conflict of Interest

The authors declare that they have no conflict of interest

Author Contributions Statement

VTH, VPS, PEF and PG contributed to experimental design, interpretation and writing. PEF supported the technique. VTH conducted the technique. TDAL and TLD provided technical assistance. KB and KLC administered questionnaires, followed patient and collected samples. SY, BA, DR and PP contributed to critically reviewing the manuscript. PG coordinated the work.

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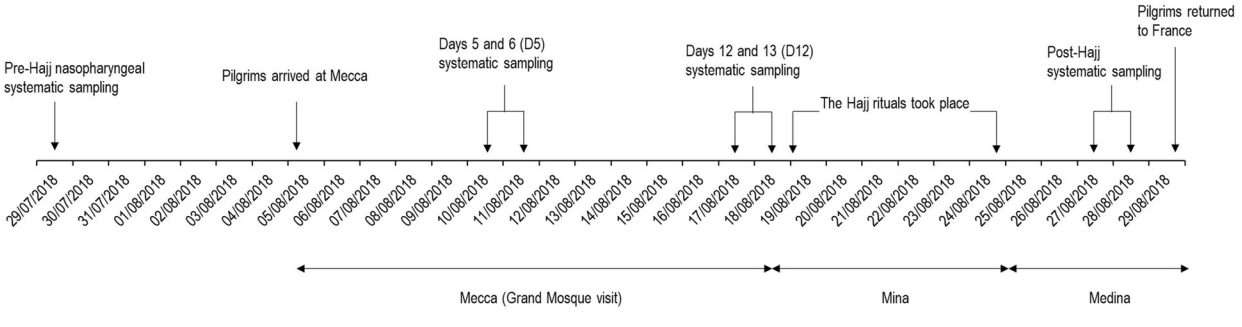
Figure 1: Study design of cohort survey on the dynamic of *Haemophilus influenzae* carriage among 121 French pilgrims during the 2018 Hajj season

Figure 2: Temporal distribution of 128 MLST positive samples among 75 pilgrims with at least one positive sample.

Figure 3: Number of MLST positive samples and sequences successfully assigned of seven MLST genes


Pre-Hajj nasopharyngeal systematic sampling
Pilgrims arrived at Mecca
Days 5 and 6 (D5) systematic sampling
Days 12 and 13 (D12) systematic sampling
The Hajj rituals took place
Post-Hajj systematic sampling
Pilgrims returned to France
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30/07/2018
31/07/2018
01/08/2018
02/08/2018
03/08/2018
04/08/2018
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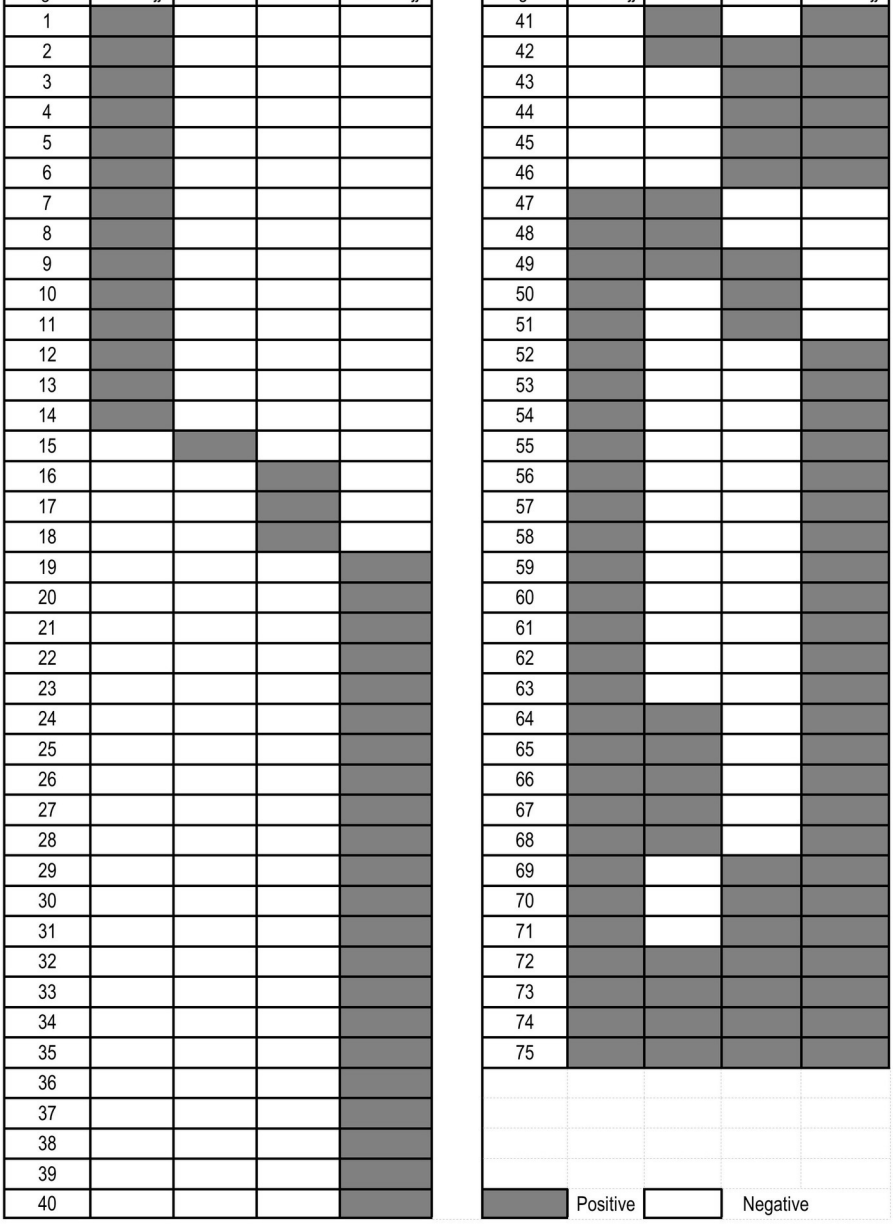
Mecca (Grand Mosque visit)
Mina
Medina


Pilgrim
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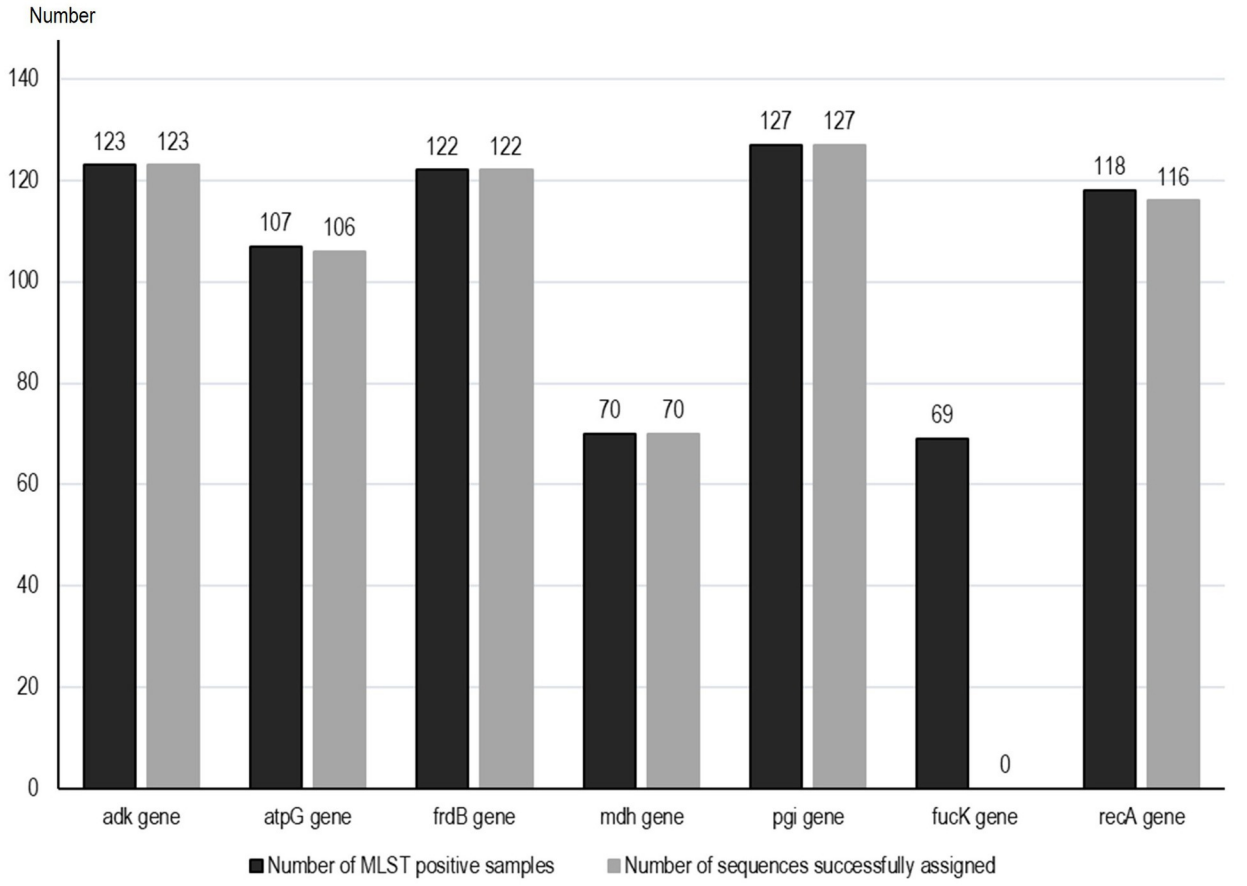
Positive
Negative


Number
140
120
100
80
60
40
20
0
123
123
107
106
122
122
70
70
127
127
69
0
118
116
adk gene
atpG gene
frdB gene
mdh gene
pgi gene
fucK gene
recA gene
Number of MLST positive samples
Number of sequences successfully assigned