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Acquisition of a High Diversity of Bacteria during the Hajj Pilgrimage, Including *Acinetobacter baumannii* with bla_{OXA-72} and *Escherichia coli* with bla_{NDM-5} Carbapenemase Genes

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Pilgrims returning from the Hajj (pilgrimage to Mecca) can be carriers of multidrug-resistant bacteria (MDR). Pharyngeal and rectal swab samples were collected from 98 pilgrims before and after they traveled to the Hajj in 2014 to investigate the acquisition of MDR bacteria. The bacterial diversity in pharyngeal swab samples was assessed by culture with selective media. There was a significantly higher diversity of bacteria in samples collected after the return from the Hajj than in those collected before ($P = 0.0008$). Surprisingly, *Acinetobacter baumannii* strains were isolated from 16 pharyngeal swab samples (1 sample taken during the Hajj and 15 samples taken upon return) and 26 post-Hajj rectal swab samples, while none were isolated from samples taken before the Hajj. Testing of all samples by real-time PCR targeting bla_{OXA-51} gave positive results for only 1% of samples taken during the Hajj, 21/90 (23.3%) pharyngeal swab samples taken post-Hajj, and 35/90 (38.9%) rectal swab samples taken post-Hajj. One strain of *A. baumannii* isolated from the pharynx was resistant to imipenem and harbored a bla_{OXA-72} carbapenemase gene. Multilocus sequence typing analysis of 43 *A. baumannii* isolates revealed a huge diversity of 35 sequence types (STs), among which 18 were novel STs reported for the first time in this study. Moreover, we also found one *Escherichia coli* isolate, collected from a rectal swab sample from a pilgrim taken after the Hajj, which harbored bla_{NDM-5}, $bla_{CTX-M-15}$, bla_{TEM-1}, and *aadA2* (ST2659 and ST181). In conclusion, pilgrims are at a potential risk of acquiring and transmitting MDR *Acinetobacter* spp. and carbapenemase-producing Gram-negative bacteria during the Hajj season.

The Hajj (pilgrimage to Mecca) is the world's largest annual mass gathering which brings together millions of people from many countries to perform rituals in Saudi Arabia. Interaction and close contact are important factors for the transmission and dissemination of infectious diseases among pilgrims. Several airborne infectious diseases have been reported during the Hajj, including meningitis, flu, and tuberculosis (1). Common respiratory pathogens have been reported among pilgrims with clinical pneumonia, including *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Acinetobacter baumannii* (2, 3) Moreover, high rates of acquisition of rhinovirus and *Streptococcus pneumoniae* as well as coronavirus E229 by pilgrims were reported during the 2013 Hajj (4). With the global spread of antibiotic-resistant bacteria, international travelers are potentially at risk of acquiring multidrug-resistant (MDR) bacteria and antibiotic resistance (AR) genes and of transferring these to other people when they return to their home countries (5, 6). This has been well documented, with reports describing the acquisition of NDM-1-producing bacteria in travelers returning from the Indian subcontinent to the United Kingdom (7) and KPC-producing bacteria in French patients who had traveled to the United States (8). We recently reported the acquisition of colistin-resistant *Salmonella enterica* serotype Newport in rectal swab samples taken from French pilgrims during the 2013 Hajj (6, 9). *Acinetobacter* species, particularly MDR *A. baumannii* strains, are primarily associated with hospital-acquired pneumonia. However, several studies have reported cases of community-acquired *A. baumannii* pneumonia, such as in Australia during the rainy season, for which the source and mode of transmission remain unknown (10). We aimed to determine, using pharyngeal and rectal swabs, whether pilgrims might have acquired MDR bacteria during the 2014 Hajj. Here we report for the first time the acquisition of cephalosporin- and imipenem-resistant *A. baumannii* and *Escherichia coli* among pilgrims returning from Saudi Arabia.

MATERIALS AND METHODS

Study design. Data were obtained between 19 September and 12 October 2014 (24 days) from a cohort of pilgrims that was traveling from France to Mecca in Saudi Arabia with a specialized travel agency in Marseille, France, for the 2014 Hajj. Inclusion in the study was voluntary, and all participants were asked to sign a consent form. The study protocol was approved by the Aix Marseille University review board and supported by a grant from the Assistance Publique-Hôpitaux de Marseille. Upon inclusion, the participants were interviewed by Arabic-speaking investigators using a standardized pretravel questionnaire that collected information on demographics. A posttravel questionnaire collecting clinical data was completed by a single investigator, who traveled with the

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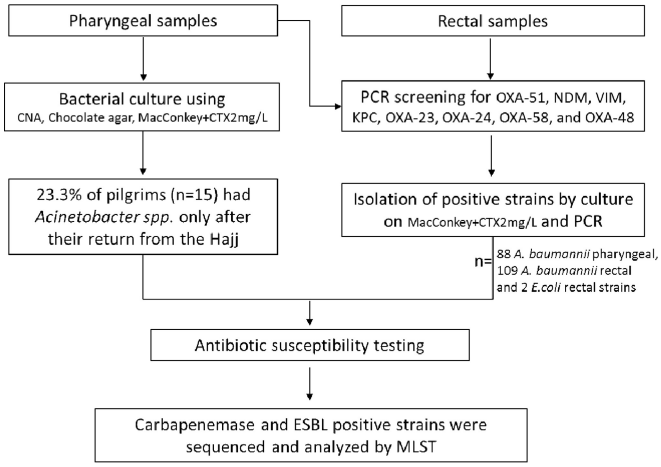

FIG 1 Flow diagram of the methods used to determine the acquisition of MDR bacteria during the Hajj. CTX, cefotaxime; CNA, colistin-nalidixic acid; ESBL, extended-spectrum β-lactam.

pilgrims, during a face-to-face interview 2 days before the return to France.

Sample collection and bacterial identification. Throat swab samples were obtained from 98 (100%), 9 (9%), and 90 (91.8%) participants pre-Hajj, during the Hajj, and post-Hajj, respectively, while rectal swab samples were collected from 92 (93.8%) pilgrims before, 1 (1%) pilgrim during, and 90 (91.8%) pilgrims upon their return from Hajj 2014. The swab samples collected from the participants were placed in viral transport medium (Transwab; Sigma) at the point of collection and kept at −20°C before being transported to the laboratory in Marseille for storage in a −80°C freezer within 48 h of collection. Next, 1.5 ml of Hanks' balanced salt solution (HBSS) for pharyngeal swab samples or phosphate-buffered saline (PBS) for rectal swab samples was added. A 250-μl volume of each sample was transferred to 8 ml of Trypticase soy broth (TSB) and incubated at 37°C overnight. A flow diagram of the methods used to determine the acquisition of MDR bacteria during the Hajj is shown in Fig. 1. At the onset, all pharyngeal swab samples were cultured to determine the bacterial diversity occurring between the pre- and post-Hajj samples by plating the samples onto Columbia colistin-nalidixic acid and chocolate agar under a 5% CO_2 atmosphere at 37°C. In addition, MacConkey agar containing 2 mg/liter cefotaxime, incubated aerobically at 37°C, was used to isolate resistant bacteria. Different types of colonies were collected to identify the bacterial species using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Microflex; Bruker Daltonics, Bremen, Germany) and flex control software (Bruker Daltonics) as previously described (11). A chi-square test was used to compare the bacterial species diversity between pre- and post-Hajj samples.

AST. Antibiotic susceptibility testing (AST) was performed on Mueller-Hinton agar using the disk diffusion method and 16 different antibiotics, including aztreonam, cefoxitin, ceftriaxone, cefotaxime, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, amoxicillin, tobramycin, gentamicin, amikacin, ciprofloxacin, ofloxacin, rifampin, imipenem, sulfamethoxazole-trimethoprim, and colistin. The MICs of imipenem and ceftriaxone were determined using the Etest method (AB Biodisk, Solna, Sweden). The results were interpreted as recommended by EUCAST (www.eucast.org).

Screening of samples by real-time PCR and molecular characterization of MDR bacteria. DNA was then extracted from the pharyngeal and rectal swab samples using an EZ1 BioRobot instrument (Qiagen S.A., Courtaboeuf, France) according to the manufacturer's instructions. Real-time PCR targeting the bla_{OXA-51} and carbapenemase (bla_{NDM-1}, bla_{OXA-23}, bla_{OXA24}, bla_{OXA-58}, bla_{OXA-48}, and bla_{VIM}) genes was performed to identify *A. baumannii* and carbapenemase-producing bacteria in the pharyngeal and rectal swab samples. The PCR-positive samples were then cultured again to isolate MDR bacteria. All nonduplicate MDR bacteria were searched for AR genes using PCR for carbapenemase and extended-spectrum β-lactamase (bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{VEB}, bla_{GES}, and bla_{PER}) genes. The primers used in this study have previously been described elsewhere (12–17). Positive PCR results were confirmed by sequencing using the BigDye Terminator chemistry on an ABI 3730 sequencer (Life Technologies, USA), and then the sequences were compared to those in the ARG-ANNOT database to identify AR genes (18).

MLST typing. Nonduplicate *A. baumannii* isolates were typed by multilocus sequence typing (MLST) as described for the Pasteur MLST database protocol (http://pubmlst.org/abaumannii/). Sequence types (STs) were assigned to the isolates by the Pasteur Institute website (on the basis of data available as of 5 November 2015). The eBURST program (version 3; http://eburst.mlst.net/) was used to assign STs to clonal complexes (CCs). A CC was assigned for each group using six as the minimum number of identical loci for the definition of a CC and three as the minimum for single-locus variants.

For bla_{NDM-5}-positive *E. coli* strains, the MLST was assigned according to the Warwick MLST *E. coli* database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

RESULTS

Demographics and clinical data. A total of 98 pilgrims were enrolled. Sixty-six (67.4%) were female and 32 (32.6%) were male, and the mean age was 61 years. The majority were of North African origin, with 63 (64.2%) having been born in Algeria and 12 (12.4%) having been born in Tunisia. For 67.4% of the pilgrims, this was their first pilgrimage to Mecca. Of the 98 pilgrims, 76 (77.5%) reported at least one respiratory symptom during the reported travel period. Among the pilgrims with respiratory or flu-like symptoms, the most frequently reported symptoms were cough (67/76 [88%], with 40/76 [53%] having a productive cough), sore throat ($n = 65$, 86%), rhinitis ($n = 56$, 74%), and loss of voice ($n = 47$, 62%). Sixteen (21%) met the criteria for influenza-like illness (ILI), defined as a cough, sore throat, and fever. Less frequently reported symptoms ($n = 54$) included shortness of breath ($n = 9$, 16.6%), fever ($n = 18$, 33.3%), muscle aches ($n = 16$, 29.6%), diarrhea ($n = 10$, 18.5%), and vomiting and conjunctivitis ($n = 1$). None of the pilgrims needed to attend a hospital for their respiratory symptoms, and all care was provided in the community. Forty-three pilgrims received antibiotics. Symptoms continued and were present upon their return for 42/76 (55%) of the pilgrims presenting with respiratory symptoms.

Bacterial diversity in pharyngeal swab samples. The study of the microbial community using culture-based techniques allowed us to isolate 70 different bacterial species from pharyngeal swab samples pre- and post-Hajj (Fig. 2). The bacterial diversity in the post-Hajj samples was significantly higher than that in the pre-Hajj samples ($P = 0.0008$). There were 35 specific species in the post-Hajj samples, particularly *Acinetobacter* species, including *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter calcoaceticus*, *Acinetobacter ursingii*, *Acinetobacter septicus*, and *Acinetobacter baylyi*. None of the pharyngeal swab samples collected from the pilgrims pre-Hajj tested positive for *Acinetobacter* spp. Thus, DNA was extracted from all the pharyngeal and rectal swab samples to identify *A. baumannii* and carbapenemase-encoding genes using PCR.

Screening for *Acinetobacter baumannii* by PCR. A total of 381 samples (pharyngeal and rectal swab samples) were screened for *A. baumannii* using real-time PCR targeting the bla_{OXA-51} gene. None of the samples taken before travel were $bla_{OXA-51-like}$ positive. In total, 2/9 (22.2%) pharyngeal swab samples collected

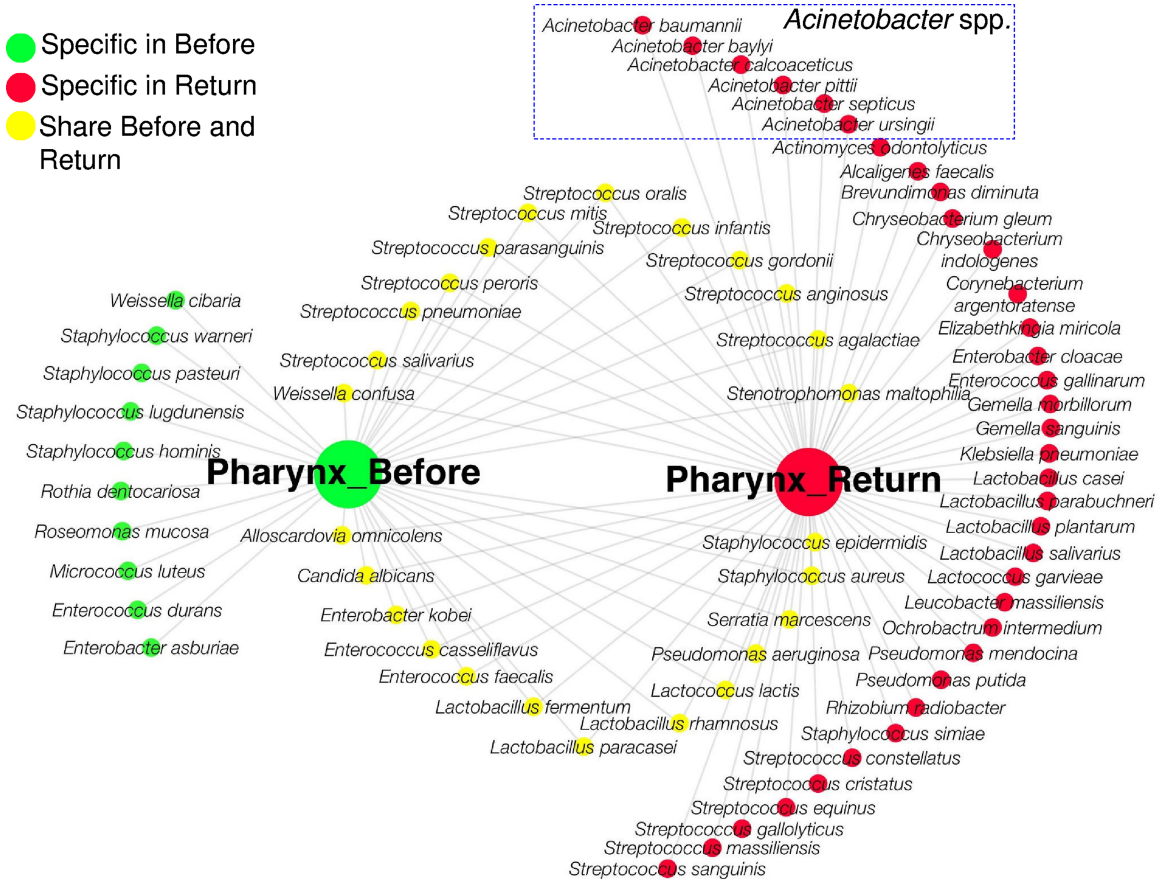

FIG 2 Bacterial species cultured from pharyngeal swab samples from pilgrims before and after they traveled to the 2014 Hajj.

during the Hajj, 21/90 (23.3%) pharyngeal swab samples collected post-Hajj, and 35/90 (38.9%) rectal swab samples collected post-Hajj were $bla_{\text{OXA-51-like}}$ positive. After reisolation of *A. baumannii* from the $bla_{\text{OXA-51-like}}$-positive samples, *A. baumannii* was recovered from 16 pharyngeal swab samples (1 of 2 samples collected during the Hajj and 15 of 21 samples collected post-Hajj) and 26 of 35 rectal swab samples. Both pharyngeal and rectal swab samples from three pilgrims were culture positive for *A. baumannii* and $bla_{\text{OXA-51-like}}$ positive.

Antibiotic susceptibility testing results for *A. baumannii*. A total of 88 *A. baumannii* colonies from pharyngeal swab samples and 109 *A. baumannii* colonies from rectal swab samples were tested (on average, 5 colonies were randomly selected from each sample) from samples collected during and after the Hajj using MacConkey agar with 2 mg/liter of cefotaxime as a selective medium. AST showed 43 different resistance profiles for the *A. baumannii* isolates (17 isolates from pharyngeal swab samples and 26 isolates from rectal swab samples). One of the pharyngeal swab samples (sample 149) contained *A. baumannii* isolates with two different AST profiles (strains 149R1 and 149R2), as shown in Fig. 3. Disk diffusion tests performed on all strains showed a ceftriaxone resistance rate of 95.35%. The MICs of ceftriaxone ranged from 1.5 to 16 μg/ml. The resistance profiles of the isolates from both the pharyngeal and rectal swab samples were identical. No isolates were found to be resistant to amikacin, rifampin, or colistin. One *A. baumannii* isolate recovered from a pharyngeal swab sample (strain 149R1) was resistant to imipenem (MIC, 8 μg/ml). The $bla_{\text{OXA-72}}$ gene was detected and confirmed by sequencing. All isolates from the rectal swab samples were susceptible to imipenem. One isolate (strain 129R) showed resistance to most antibiotics; the exceptions were amikacin, rifampin, imipenem, and colistin. This strain harbored $bla_{\text{TEM-1D}}$.

Sequence diversity of *A. baumannii* isolates. To compare the genotypic diversity of the *A. baumannii* isolates in pharyngeal and rectal swab samples, MLST was used to identify sequence types (STs) and clonal complexes (CCs). A total of 35 unique STs were identified from 43 nonduplicate *A. baumannii* isolates (17 isolates from pharyngeal swab samples and 26 isolates from rectal swab samples). Of the 30 STs found in a single isolate, 18 were reported to be new STs by MLST Pasteur: ST758 to ST775 (Fig. 3).

A population snapshot of the *A. baumannii* isolates obtained in our study is shown in Fig. 4. Six new STs were grouped in CC2 with the known ST239. Other STs were scattered across different CCs, including CC3, CC10, and CC25. Comparing the STs of the *A. baumannii* isolates between pharyngeal and rectal swab samples, three isolates were found to be ST10, with two of these strains being isolated from the pharyngeal and rectal swab samples of the same pilgrim (strains 149R2 and 149R3) and one being isolated from the rectal swab sample of pilgrim 55. Several STs were found in at least two strains, such as ST49 (pharynx, strains 7R and 84R), ST150 (pharynx, strains 47R and 59R; rectum, strain 57R), ST241

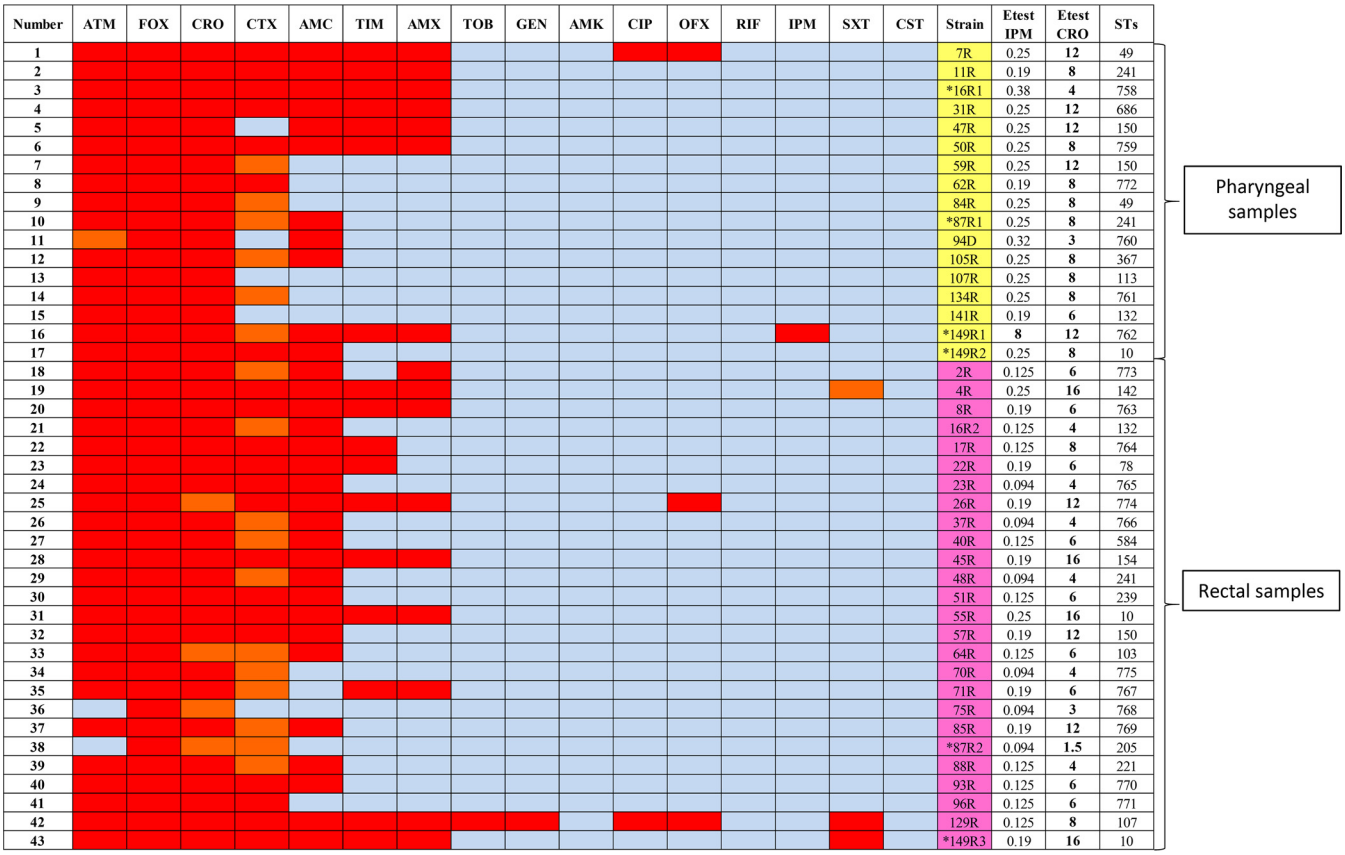

FIG 3 Antibiotic susceptibility profiles and STs of 43 *Acinetobacter baumannii* isolates recovered from pharyngeal and rectal swab samples from pilgrims returning from Hajj 2014. Red squares, resistance; blue squares, susceptibility; orange, intermediate. The MIC values of imipenem and ceftriaxone are provided for each isolate. ATM, aztreonam; FOX, cefoxitin; CRO, ceftriaxone; CTX, cefotaxime; AMC, amoxicillin-clavulanic acid; TIM, ticarcillin-clavulanic acid; AMX, amoxicillin; TOB, tobramycin; GEN, gentamicin; AMK amikacin; CIP, ciprofloxacin; OFX ofloxacin; RIF, rifampin; IPM, imipenem; SXT, sulfamethoxazole-trimethoprim; CST, colistin. The strain number represents the pilgrim's number and the source of sample (D, during Hajj; R, upon return [i.e., post-Hajj]). Samples containing two different bacteria are indicated by the number after the source of the sample. Asterisk, strains isolated from the same pilgrim.

(pharynx, strains 11R and 87R; rectum, strain 48R), and ST132 (pharynx, strain 141R; rectum, strain 16R).

Detection of carbapenemase genes in samples. Carbapenemase genes were detected in the samples as follows: $bla_{\text{OXA-72}}$ in 1 post-Hajj pharyngeal swab sample (strain 149R1), bla_{NDM} in 2 post-Hajj rectal swab samples (pilgrim 4, $bla_{\text{NDM-5}}$; pilgrim 22, $bla_{\text{NDM-1}}$), $bla_{\text{OXA-48}}$ in pre- and post-Hajj samples from pilgrim 78, and $bla_{\text{OXA-58}}$ in 22 post-Hajj samples (3 pharyngeal swab samples, 19 rectal swab samples). Carbapenem-resistant bacteria were reisolated from the samples positive by PCR. We found that *A. baumannii* harbored both $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-72}}$ genes. We recovered *E. coli* with $bla_{\text{NDM-5}}$ from only one post-Hajj sample that was PCR positive (pilgrim 4). This sample contained two types of *E. coli* strains: strain P5 (ST2659) harboring $bla_{\text{NDM-5}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, and *aadA2* and strain P9 (ST181) harboring $bla_{\text{NDM-5}}$, $bla_{\text{TEM-1}}$, and *aadA2*. These two *E. coli* strains were susceptible to colistin and imipenem, but strain P9 was also susceptible to aztreonam, ciprofloxacin, and ofloxacin. In our study, we were unable to isolate $bla_{\text{OXA-48}}$- and $bla_{\text{OXA-58}}$-positive strains from the samples.

DISCUSSION

MDR bacteria are becoming a major public health concern. They can spread to different environments through close contact, food, water, or animals (19, 20). The Hajj in particular is well-known to be a source of transmission of infectious diseases in the pilgrims' countries of origin (1, 2, 4). Our findings highlighted that pilgrims showed a higher diversity of bacteria, particularly *Acinetobacter* spp., upon their return, and acquired carbapenemase-producing *A. baumannii* and *E. coli* during the 2014 Hajj. The high rate of acquisition of a high diversity of bacteria during the Hajj may be due to the overcrowded conditions, illustrated by the high frequency of respiratory symptoms suggesting human-to-human transmission and the comorbidities facilitating respiratory tract infection. This pharyngeal microbiome may change during an individual's life as a result of the foods and pharmaceutical products that the individual consumes or environmental exposure. *A. baumannii* has been increasingly documented as an important cause of community-acquired pneumonia in several countries, including Australia, Taiwan, Singapore, and Saudi Arabia (2, 10, 21, 22).

During the ritual period, 77.5% of pilgrims reported at least one respiratory symptom. Asthma and respiratory tract infections might contribute to the spread of inhaled microbes (23). Moreover, there was the possible effect of desert dust and other particles in the spread of airborne bacteria (24). Also, the "Hajj cough" has been reported to be a highly common symptom among pilgrims at religious places (25). The pilgrims' high rates of antibiotic con-

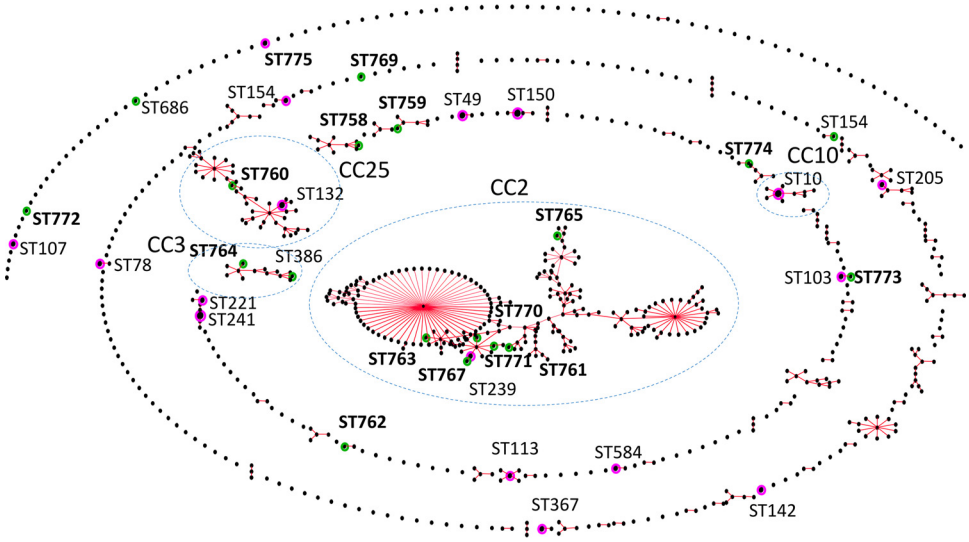

FIG 4 eBURST analysis (http://eburst.mlst.net) comparing the tested strains with all of the STs in the *Acinetobacter baumannii* MLST database (Pasteur scheme; 776 STs; http://pubmlst.org/abaumannii/; last accessed 5 November 2015). The STs of 43 *A. baumannii* strains referred to in the text are indicated. Bold font indicates new STs described in this study. The circles indicate that our STs belonged to a CC.

sumption should potentially be considered a consequence of respiratory infections, introducing the possibility of selection of resistant strains (25). However, the dissemination and acquisition of *Acinetobacter* spp. would occur via community contact between pilgrims, and in particular, carbapenemase genes would be transmitted among bacteria via plasmids.

A. baumannii isolates carrying $bla_{\text{OXA-72}}$ were found in pharyngeal swab samples in our study. This carbapenemase gene has recently been reported in a plasmid and has led to imipenem resistance among isolates from patients in Taiwan (26). Interestingly, the $bla_{\text{OXA-72}}$ carbapenemase has recently been reported in a patient in Saudi Arabia (27). The transmission of resistance genes from person to person or from the environment to people might easily occur. In general, *Acinetobacter* spp. can be isolated from several sources, including soil contaminated with petroleum hydrocarbons, vegetables, aquaculture farms, and animals (28). Moreover, *Acinetobacter* spp. are able to colonize inanimate surfaces and equipment, such as staff uniforms, benchtops, and trolleys (29). These results show that *Acinetobacter* spp. are able to tolerate a range of different conditions and *Acinetobacter* spp. may be disseminated through the air, food, and close contact. The colonization of pilgrims with *Acinetobacter* spp. might have been transient, but in the absence of follow-up this cannot be ascertained. Thus, we cannot speculate on the origin of the colonization. Some of our *A. baumannii* strains were hospital associated, including ST10 (international clone 8), CC25 (international clone 7) strains (30, 31), while the imipenem-resistant strain belonged to a new ST (ST762).

Some pilgrims who were colonized by *Acinetobacter* spp. took antibiotics during the Hajj. Antibiotic intake may have played a role by selecting *Acinetobacter* spp. resistant to β-lactams. However, pilgrim 149, who carried *A. baumannii* ST10 and imipenem-resistant strains, did not take any antibiotics during the pilgrimage. Moreover, the MLST results showed a huge diversity of *A. baumannii* isolates, and it is possible that during the Hajj the pilgrims acquired *Acinetobacter* species from different sources and in different ways, including by community contact through microaspiration (10); during rituals, such as when they kissed or touched the stone; and through food and water consumption. Additionally, the same *A. baumannii* STs were found in both pharyngeal and rectal swab samples collected from the same pilgrim. This demonstrates that bacteria acquired by pilgrims can transfer from the upper to the lower digestive tract.

However, the pilgrims acquired not only *Acinetobacter* species but also *E. coli* isolates with $bla_{\text{NDM-5}}$. The $bla_{\text{NDM-5}}$ resistance gene has been reported in *E. coli* and *Klebsiella pneumoniae* isolates from hospitalized patients in several countries, including Denmark, South Korea, India, Algeria, the United Kingdom, Japan, and Spain (32–44), and from a dog in Algeria (45). One of our *E. coli* isolates possessed an ST2659 sequence previously recovered between January 2012 and February 2013 from urine and blood specimens from patients in the University Hospital of Annaba (east Algeria) (34). This pilgrim's pre-Hajj sample, however, was negative by PCR and culture. This indicated that this pilgrim acquired $bla_{\text{NDM-5}}$ during the Hajj and could have acted as a reservoir when he returned to his country.

The high level of acquisition of MDR *Acinetobacter* spp. and *E. coli* described here is of concern. Pilgrims may carry these bacteria and transfer them to others when they return to their countries. The source and mode of transmission in the community are still not understood. Personal hygiene should be taught and monitored to prevent and reduce the rate of MDR bacterial transmission.

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