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The molecular characterisation of bacteria associated with neonatal necrotising enterocolitis and sepsis which were isolated from hospitals in Bogotá, Colombia

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Abstract

Background: The identification and molecular characterization of bacteria associated with neonatal necrotising enterocolitis (NNE) and sepsis is very important in clarifying the role such bacteria play in the development of this disease.

Methods: The present multicentre study was aimed at characterizing bacteria isolated from haemocultures obtained from 20 neonates suffering NNE by using traditional and molecular methodologies (16 S rRNA subunit sequencing and multilocus sequence typing - MLST).

Results: NNE incidence in hospitals in Bogotá was also estimated, finding rates similar to and higher than those reported in the literature (1 to 3 cases of NNE per 1,000 live-births). *Staphylococcus epidermidis* ST2, ST81, and ST126 sequence types were identified by using these two molecular techniques; the *Escherichia coli* ST394 sequence type was also identified. Species could not be identified for the *Pantoea agglomerans* isolate due to the high degree of intra-species identity. Interestingly, the bacterial isolates from the two neonates who died were classified in the same sequence type (i.e. *S. epidermidis* ST81), even though they came from different hospitals.

Conclusion: Such molecular techniques allow characterizing bacterial pathogen populations occurring in one or more hospitals in a particular city or determined geographical area and support taking more specific preventative measures directed against such particular clones.

Key words: Necrotising enterocolitis (NNE), Sepsis, Molecular characterization, Multilocus sequence typing (MLST).

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Introduction

Necrotising enterocolitis (NNE) is the most important gastrointestinal disease affecting premature new-born who have inherent risk factors such as the immaturity of their intestines and feeding habits and unsuitable bacterial colonisation (1). Even though several microorganisms, including viruses, fungi and bacteria, have been obtained from new-born suffering necrotising enterocolitis, this disease's causal agents have not been identified to date (2). Few studies have been carried out concerning the molecular characterisation of associated bacteria as new molecular methodologies (i.e. metagenomics) are being introduced, allowing the sequencing of complete genomes from different clinically-relevant bacterial communities, such as those involved in developing necrotising enterocolitis.

The bacteria usually associated with this disease would be *Klebsiella*, *Clostridium*, *Staphylococcus spp* and *Escherichia coli*. The intestines of premature new-born infants promote colonisation by abnormal flora due to several factors such as gastric pH having a lower degree of acidity, low proteolytic enzyme concentration, little motility and alteration of the carbohydrates in the intestinal mucosa (2).

Molecular methodologies, particularly 16S rRNA subunit sequencing and multilocus sequence typing (MLST) (3), are better than conventional ones in identifying and characterising the bacteria associated with NNE because they are quicker, more precise and reproducible. The MLST technique facilitates characterising and identifying clones in bacterial pathogen populations by sequencing fragments of several constitutive genes and identifying a specific bacterial isolate's allele profiles. It is rapid because it does not require large amounts of bacterial culture, but rather small, easily obtained samples, it is precise because it is based on DNA sequences from at least seven constitutive genes present in all bacteria and reproducible as it can be repeated on more than one occasion by the same laboratory or different laboratories, the same results always being obtained (4-6).

Conventional bacterial identification methods, such as biochemical tests, morphological studies and antibiotic susceptibility tests, are only valid for bacteria which can be cultured (around 20%) (7); the rest (the great majority) require advanced molecular biology methods for identifying them and then characterising them. For example, amplifying DNA fragments from the ribosomal RNA gene's small subunit (16S rRNA) has led to identifying microorganisms which were previously difficult to classify (4) because they needed very special culture mediums and conditions to be grown in.

On the other hand, molecular epidemiology is becoming increasingly important in suitably identifying interspecies differences in bacterial populations using techniques such as pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLPs) and multilocus sequence typing (MLST). The latter methodology allows unequivocally detecting polymorphisms in constitutive gene sequences which are useful for identifying bacterial pathogen population clones according to their allele profiles, thereby ascertaining the types of sequence to which they belong (7).

The main purpose of this work was thus to study NNE and sepsis by using clinical, microbiological and molecular tools. 16 S rRNA sequencing was particularly used for bacterial identification and MLST for looking for genetic polymorphisms in constitutive genes and thereby determine allele profiles from bacterial clones or types of sequence.

Materials and Methods

Neonates' clinical characteristics. The study involved new-born who were admitted to the neonatal intensive care unit (NICU) of six hospitals in Bogotá during a one-year period. The inclusion criteria consisted of premature or full-term neonates having suspected or clinical confirmation of NNE according to the criteria established by Bell (8) with or without sepsis. Neonates suffering congenital gastric abnormalities (such as omphalocele and gastroschisis) were not included.

The project was duly reviewed and approved by the Universidad del Rosario School of Medicine and Health Sciences' Ethics Committee. The relevant information for the project was obtained from clinical histories by using a data-collection instrument recording identification data, diagnostic test results, antibiotic treatment, clinical laboratory exam results and some of the mothers' clinical characteristics.

Bacterial isolates. The bacteria isolated from positive haemocultures were identified by each hospital's conventional automated (MicroScan and Vitek) clinical laboratory methods, bearing morphological and biochemical characteristics in mind, as well as antibiotic susceptibility patterns. The samples were then taken to the Universidad del Rosario's Microbiology Unit where they were cultured in blood agar in a 5% to 7% carbon dioxide atmosphere at 37°C for 24 hours to confirm their purity and viability. They were also suspended in brain-heart infusion (BHI) broth with 15% glycerol and stored at –70°C to create a strain-bank.

Extracting and purifying genomic DNA. Two passes in blood agar were made after having been frozen in the strain-bank to recover the microorganisms and extract genomic DNA, working with a $6x10^8$ ufc/ml bacterial suspension, using turbidimetry. Genomic DNA was extracted from each isolate using around 1 ml bacterial suspension having 17 hours growth with a Dynabeads kit (DNA DIRECT, Universal system, Invitrogen, USA) for Gram-negative bacteria and a MasterPure kit (Gram-positive DNA purification kit, Epicentre, USA) for Gram-positive bacteria, following the manufacturers' recommendations. DNA was quantitatively evaluated by spectrophotometry using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), determining optical density at 260 and 280

wavelengths (λ) to ascertain purity. 1% agarose gels were also run to verify the integrity of this genomic DNA.

Identifying and characterising bacterial isolates. A 996 base pair (bp) fragment of the *E. coli* 16S rRNA gene was amplified by PCR for genotypic identification of the bacterial isolates, using previously-described U1 and U2 universal primers (9). The U1 primer (5'-GTGCCAGCAGCCGCGGTAATACG-3') covered 16SrRNA gene nucleotides 515 to 537 and the U2 primer (5'-TACCTTGTTACGACTTCACCCCA-3') covered nucleotides 1485 to 1507. The PCR reactions involved mixing 1µl genomic DNA, 10µl AmpliTaq GoldFast PCR Master Mix (2x) and 1 µl each of primer U1 and U2 (10 µM), at 20 µl final volume. PCRs conditions were as follows: initial denaturing at 95°C for 10 min, followed by 35 cycles at 96°C for 3s, annealing at 65°C for 3s an extension step at 68°C for 30s and final elongation at 72°C for 10s. The PCR products so obtained were purified and sequenced in both directions and the sequences obtained were compared to bacterial and archaeal small-subunit 16s rRNA sequence databases for identifying genera and species.

Identification by multilocus sequence typing (MLST). The allele profiles and multilocus sequence types were determined according to the protocols described in the corresponding databases for MLST. Seven constitutive genes were amplified by PCR for *S. epidermidis* (http://*sepidermidis*. mlst.net/): carbamate quinase (*arcC*), shikimate dehydrogenase (*aroE*), ABC transporter (*gtr*), DNA repair protein (*mutS*), protein pyrimidine operon regulator (PyrR), triose phosphate isomerase (*tpiA*) and acetyl-coenzyme A acetyltransferase (yqiL). Seven constitutive genes were also amplified for *E. coli* (http://mlst.ucc.ie/mlst/dbs/*Ecoli*/): adenylate kinase (*adk*), fumarate hydrate (*fumC*), DNA gyrase (*gyrB*), isocitrate dehydrogenase (icd), malate dehydrogenase (mdh), adenyl succinate dehydrogenase (*purA*) and ATP/GTP-binding motif (*recA*).

The expected PCR products were purified and then sequenced in both directions to confirm the possible variations in the sequences; FinchTV and Clustal W software was used to analyse them and then they were compared to the allele sequences reported in the databases and the sequence type of each isolate was determined (Table: 1).

Results

The estimated NNE incidence rate in hospital 2 was around 1 case per 1,000 live-births; the rate for hospital 4 was below this range, whilst hospitals 1 and 3 had 6 and 4 cases per 1,000 live-births respectively (Table 1). No neonates born in hospitals 5 and 6 developed NNE and they are thus not shown in Table 1. There was only one case of NNE in hospital 5 involving a new-born remitted from another hospital (Table 2); no NNE cases were remitted from other hospitals to hospital 6.

Table 2. Estimated NNE incidence for each participating hospital *

Hospital	Number of live-births	NNE
Hospital 1	1,032	6
Hospital 2	2,307	2
Hospital 3	1,044	4
Hospital 4	2,850	1
Total	7,233	14

* Neonates remitted from other hospitals were not taken into account, only those born in each particular hospital

A total of 19 premature and full-term neonates were included in the study during the year it lasted, 15 of whom had sepsis (6 early onset, 9 late onset and 2 both) (Table 2) and only 6 were positive in haemoculture (31%). Four neonates from hospital 1 did not have sepsis and one of the four presenting sepsis had both early and late onset neonatal sepsis. One of the three neonates from hospital 3 had early and late onset neonatal sepsis. Five neonates were classified as being stage 3 (i.e. advanced enterocolitis) and the remaining 14 as stage 2 or confirmed enterocolitis (Table: 3).

Table 3 summarises the neonates' main clinical and diagnostic characteristics. Twelve were female and 8 male; eleven of them developed NNE during the first week of life (57%). Regarding how they were fed, 17 neonates (89%) received enteral feeding, 7 (36%) parenteral and 6 (31%) both types of feeding. Regarding gestational age, 8 neonates were full-term (i.e. 37 or more weeks' gestation), 8 were preterm (having less than 34 weeks' gestation) and 4 were late-stage pre-term (34 to 36 weeks' gestation). Regarding birth-weight, 7 weighed less than 1,500 g and the remaining 13 (68%) weighed 1,630 to 3,170 g (Table: 4).

The present study's results showed that presentation of NNE was mainly characterised by symptoms such as intolerance concerning the oral route in 14 cases (73%), blood in faeces in 13 cases (68%), abdominal distension in 17 cases (89%), distended bowel loops and intestinal pneumatosis in all cases.

Conventional and molecular identification of bacterial isolates. The bacteria identified by the respective hospitals' clinical laboratories were *Staphylococcus epidermidis* (hospitals 1, 2, 4 and 5), *Escherichia coli* (hospital 1) and *Pantoea agglomerans* (hospital 1). The last mentioned isolate could not be identified at species level using 16 S rRNA sequencing due to the high identity of species forming the *P. ananatis*, *P. eucrina* and *P. dispersa* complex. The other isolates proved to be the same using both methods (Table 5). All *S. epidermidis* isolates were methicillin-resistant and the *Pantoea spp* isolate was sensitive to all antibiotics used in the susceptibility tests (Table 4). *S. epidermidis* ST81 was identified in the 2 neonates who died due to advanced enterocolitis (stage I3) and sepsis following molecular characterisation by MLST (Table 7); the first neonate was hospitalised in hospital 5 and the second in hospital 1

MLST analysis of *S.epidermidis* and *E.coli*. Figure 2 shows PCR amplification of *S. epidermidis* (the same for the 4 isolates) and *E. coli* constitutive genes. The DNA from these amplified fragments was sequenced by PCR and the results compared to MLST databases Table 6 shows the resulting allele profiles and sequence types. Two *S. epidermidis* isolates belonged to the same sequence type (ST81) and, coincidentally, were isolated from the neonates who died. The other *S. epidermidis* isolates corresponded to ST126 and ST2 and that from *E.coli* was identified as ST394.

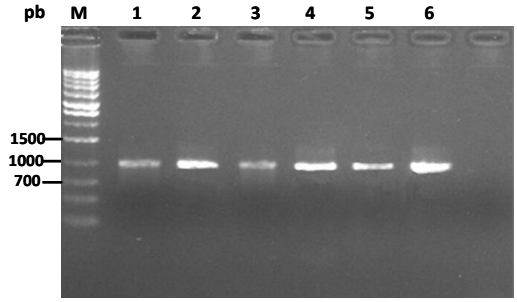

Fig. 1 PCR amplification of a 996 bp fragment from 16S rRNA gene bacterial isolates. M: molecular weight marker (1 kb), lane 1: *S. epidermidis* (hospital 5), lane 2: *S. epidermidis* (hospital 1), lane 3: *Pantoea spp.* (hospital 1), lane 4: *S. epidermidis* (hospital 2), lane 5: *E. coli* (hospital 1) and lane 6: *S. epidermidis* (hospital 4).

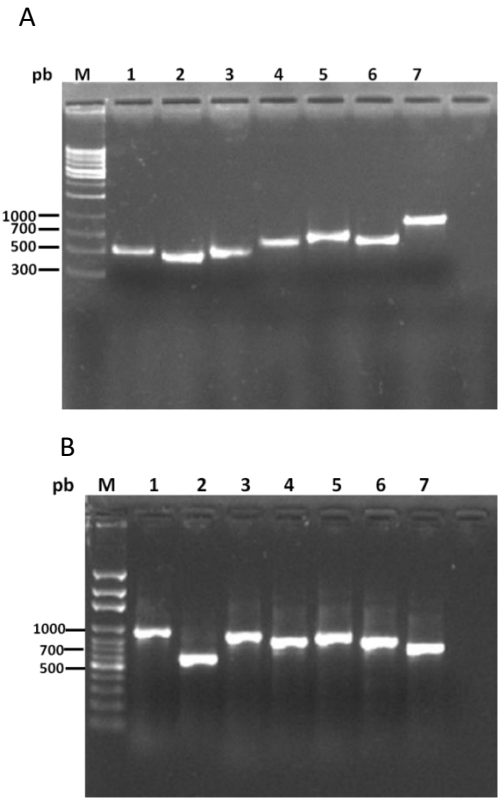

Fig. 2. PCR products from the 7 constitutive genes used for MLST analysis of *S. epidermidis* (Panel A) and *E. coli* (Panel B). M: molecular weight marker (1 kb). Panel A. lane 1: *arc*C gene, lane 2: *aro*E gene, lane 3: *gtr* gene, lane 4: *mut*S gene, lane 5: *yqi*L gene, lane 6: *tpi*A gene and lane 7: *pyr*R gene. Panel B. lane 1: *mdh* gene, lane 2: *adk* gene, lane 3: *gyrB* gene, lane 4: *fumC* gene, lane 5: *icd* gene, lane 6: *purA* gene and lane 7: *recA* gene. Gels were run on 1% agarose for one hour at 80 volts using 1x TBE as run buffer.

Discussion

In spite of already-existing preventative measures and specific treatment being available for controlling neonatal necrotising enterocolitis in Bogotá's hospitals, the estimated incidence for four of the hospitals in this study demonstrated that this was much higher than that reported in the literature (1 case per 1,000 live-births) in two hospitals, similar in one hospital and less in another hospital. The study also showed that NNE could be fulminant in some cases, resulting in death as in the case of the two neonates in whom the same bacterial isolate sequence type was coincidentally identified by MLST (i.e. *S. epidermidis* ST81). Even though prematurity is the main risk factor for developing NNE, a figure double that reported in the literature (13%) was presented in full-term neonates in 8 of the 19 cases (42%) in this study (1).

E.coli was isolated in one of the hospitals in a neonate who had late-onset sepsis, thereby confirming that this bacterial specie is one of the most frequently associated in these cases (10) and also one of the most commonly found in cases of NNE (11). MLST analysis revealed that this *E. coli* isolate was ST394, which has been reported as being enteroaggregative *Escherichia coli* (EAEC), causing diarrhoea in Nigeria and being resistant to ampicillin, tetracycline and trimethoprim (12).

In line with that reported in the literature, most *Staphylococcus epidermidis* isolates in the present study were not just associated with NNE but also with cases of late-onset neonatal sepsis (13). Even though this specie could produce a toxin similar to *Staphylococcus aureus* delta-toxin which is responsible for intestinal necrosis and haemorrhages (14), *S. epidermidis* is also related to nosocomial infections (15) thereby explaining the four isolates found in the hospitals involved in this study. Interestingly, the two neonates who died during the study were infected with *S. epidermidis* presenting the same sequence type (ST81) but coming from two different hospitals (hospitals 1 and 5). ST81 has already been reported in neonatal intensive care units in Estonia (16). The other two *S. epidermidis* sequence types (ST126 and ST2, isolated in hospitals 2 and 4, respectively) have already been reported in the literature. ST126 has been associated with an aggressive and virulent clinical course in several diseases and ST2 has been found in 13 countries to date; the latter ST is resistant to several drugs and has been found in different intra-hospital settings around the world (17, 18).

The bacterial isolate from hospital 1 which was identified as *Pantoea spp.* has also been associated with NEC in three worldwide case reports (19, 20, 21), as well as being involved in septicaemia and the infection of catheters and medical devices (٢٢). Even though the *Pantoea* specie could not be identified by 16SrRNA sequencing, as the *agglomerans* specie belongs to a complex strictly related to *P. eucrina*, *P. dispersa* and *P. ananatis*, this problem could become resolved by sequencing the repA gene (replication protein) and tests for biochemical characteristics such as such as phosphomycin resistance and D-tartrate use (2٣).

Conclusions

Even though the number of samples collected and the period of time this study lasted were not optimum, it did lead to demonstrating that cases of NNE are still occurring in some neonatal intensive care units in hospitals in Bogotá, in spite of already-existing prevention and control measures having been taken.

It is very important that the aforementioned molecular biology techniques are introduced in Colombian hospitals' microbiology laboratories to ascertain which bacterial pathogen population clones or sequence types are circulating, thereby enabling more specific preventative measures directed against these particular clones to be taken.

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Table 1. *S. epidermidis* and *E. coli* constitutive gene primers

Bacteria and genes	Primer sequence (5'→3') Sense	Antisense	Size (bp)
S. epidermidis			
*arc*C	TGTGATGAGCACGCTACCGTTAG	TCCAAGTAAACCCATCGGTCTG	465
*aro*E	CATTGGATTACCTCTTTGTTCAGC	CAAGCGAAATCTGTTGGGG	420
gtr	CAGCCAATTCTTTTATGACTTTT	GTGATTAAAGGTATTGATTTGAAT	438
*mut*S	GATATAAGAATAAGGGTTGTGAA	GTAATCGTCTCAGTTATCATGTT	412
*pyr*R	GTTACTAATACTTTTGCTGTGTTT	GTAGAATGTAAAGAGACTAAAATGAA	428
*tpi*A	ATCCAATTAGACGCTTTAGTAAC	TTAATGATGCGCCACCTACA	424
*yqi*L	CACGCATAGTATTAGCTGAAG	CTAATGCCTTCATCTTGAGAAATAA	416
	E. coli		
adk	ATTCTGCTTGGCGCTCCGGG	CCGTCAACTTTCGCGTATTT	583
*fum*C	TCACAGGTCGCCAGCGCTTC	GTACGCAGCGAAAAAGATTC	806
*gyr*B	TCGGCGACACGGATGACGGC	ATCAGGCCTTCACGCGCATC	911
icd	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	GGACGCAGCAGGATCTGTT	878
mdh	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	932
*pur*A	CGCGCTGATGAAAGAGATGA	CATACGGTAAGCCACGCAGA	816
*rec*A	CGCATTCGCTTTACCCTGACC	TCGTCGAAATCTACGGACCGGA	780

Table 3. Confirmed cases of NNE associated with sepsis*

Hospital	NNE	Sepsis			No sepsis
		Early-onset	Late-onset	Both	
Hospital 1	7	1	3	1	4
Hospital 2	5	2	3	0	0
Hospital 3	3	1	1	1	0
Hospital 4	3	1	2	0	0
Hospital 5	1	1	0	0	0
Hospital 6	0	0	0	0	0
Total	19	6	9	2	4

* Neonates born in this hospital and those remitted from other institutions

Table 4. The main clinical characteristics were evaluated in neonates suffering NNE

Characteristics	Total number of neonates (%)	Haemoculture	
		Positive	Negative
Neonates	n=19	n=6	n=13
NNE diagnosed (first week of life)	11(57)	3(50)	8(57)
Male	8(42)	2(33.3)	6(42.8)
Female	12(63)	4(67)	8(57)
Bell's clinical stage (NNE 3)	5(26)	3(50)	2(14.2)
Pneumatosis intestinalis (PI)	19(100)	6(100)	14(100)
Pneumoperitoneum	3(15)	2(33.3)	1(7.2)
Leukopenia	6(31)	2(33.3)	4(28.5)
Neutropenia	11(57)	5(83)	6(42.8)
Thrombocytopenia	8(42)	2(33.3)	6(42.8)
Surgery (resection)	6(31)	2(33.3)	4(28.5)
Surgery (laparotomy)	5(26)	1(16.7)	4(28.5)
Deaths	2(10)	2(33.3)	0(0)
Earl-onset sepsis (<72h)	6(31)	1(16.7)	5(36)
Late-onset sepsis (>72h)	12(63)	5(83)	7(50)
Enteral feeding	17(89)	4(67)	13(93)
Parenteral feeding	7(36)	3(50)	6(42.8)
Hypoglycaemia	7(36)	1(16.7)	6(42.8)
RDS [1]	2(10)	0(0)	2(14.2)
RPOM [2]	5(26)	2(33.3)	3(21.4)
Antenatal steroids	7(36)	3(50)	4(28.5)
Birth-weight (<1500g)	7(36)	3(50)	4(28.5)
(1630 g-3170 g)	13(68)		
Gestational age (>37 weeks)	8(42)	3(50)	3(21.4)
(34-36 week)	4(21)		
Caesarean birth	12(63)	3(50)	8(64.2)
Apgar score (low at 1 min after birth)	3(15)	1(16.7)	2(14.2)
Apgar score (low at 5 min after birth)	1(5)	0(0)	1(7.2)
Hypoxia	5(26)	2(33.3)	3(21.4)
Supplied with antibiotics (>10 days)	6(31)	2(33.3)	4(28.5)
Mechanical ventilation	11(57)	5(83)	6(42.8)
Umbilical catheterisation	3(15)	2(33.3)	1(7.2)
Epicutaneous catheterisation	19(100)	6(100)	13(100)

[1] RDS = respiratory distress syndrome [2] RPOM = premature rupture of the membranes

Table 5. Antibiotic therapy and the isolates' antimicrobial resistance

Isolate	Antimicrobial resistance
S. epidermidis (hospital 5)	Amoxicillin/clavulanate, ampicillin/sulbactam, ceftriaxone, ampicillin, erythromycin, gentamycin, oxacillin, penicillin, tetracycline.
S. epidermidis (hospital 1)	Amoxicillin/clavulanate, ceftriaxone, erythromycin, gentamycin, oxacillin
P. agglomerans (hospital 1)	None
S. epidermidis (hospital 2)	Ampicillin/sulbactam, erythromycin, clindamycin, oxacillin, penicillin
E.coli (hospital 1)	Ampicillin, piperacyline, tetracycline, trimethoprim/sulfamethoxazole
S. epidermidis (hospital 4)	Amoxicillin/clavulanate, ampicillin/sulbactam, ceftriaxone, ciprofloxacin, clindamycin, erythromycin, gentamycin, levofloxacin, oxacillin, trimethoprim sulfamethoxazole

Table 6. Identification of NNE-associated bacteria

Origin of isolate	Clinical laboratories in each hospital	Microbiology unit, UR*
Hospital 5	*S. epidermidis*	*S. epidermidis*
Hospital 1	*S. epidermidis*	*S. epidermidis*
Hospital 1	*Pantoea agglomerans*	*Pantoea dispersa* *Pantoea ananatis* *Pantoea eucrina*
Hospital 1	*Escherichia coli*	*Escherichia coli*
Hospital 2	*S. epidermidis*	*S. epidermidis*
Hospital 4	*S. epidermidis*	*S. epidermidis*

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Table 7. MLST identification of bacterial isolates' allele profiles and sequence types (ST) in the present study

Species	Constitutive genes	Allele profile	Sequence type (ST)
S. epidermidis (hospital 5)	*arcC, aroE, gtr, mutS, pyrR, tpi, yqiL*	2-17-1-1-2-1-1	81
S. epidermidis (hospital 1)	*arcC, aroE, gtr, mutS, pyrR, tpi, yqiL*	2-17-1-1-2-1-1	81
S. epidermidis (hospital 2)	*arcC, aroE, gtr, mutS, pyrR, tpi, yqiL*	7-1-1-1-3-1-1	126
S. epidermidis (hospital 4)	*arcC, aroE, gtr, mutS, pyrR, tpi, yqiL*	7-1-2-2-4-1-1	2
E.coli (hospital 1)	*adk, fum*C, *gyr*B, *icd, mdh, pur*A, *rec*A	21-35-61-52-5-5-4	394

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