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Characteristics of a Capnophilic Small Colony Variant of *Escherichia Coli* Co-Isolated With Two Other Strains From a Patient With Bacteremia in China

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Abstract

Small colony variants (SCVs) are a slow-growing subpopulation of bacteria characterized by their atypical colony morphology and distinct biochemical properties, which are known to cause chronic persistent infections. Here, we investigated the characteristics of three phenotypes of *Escherichia coli*, including a capnophilic SCV, co-isolated from a 64-year-old patient with bacteremia in China. The three strains were identified as a capnophilic strain (EC1), a capnophilic SCV (EC2), and a wild-type strain (EC3). The EC1 and EC2 strains did not grow in the absence of CO_2, while the EC2 colonies were pinpoint in appearance and had the ability to revert to the large-form phenotype. The growth of the SCV was slow and not enhanced in the presence of thymidine, hemin, thiamine, and menadione. The results of antimicrobial susceptibility showed similar sensitivity to cefoxitin and imipenem, but resistant to most of the other antimicrobials tested. Whole genome sequencing showed that no genetic mutational variations associated with SCVs were observed, while EC1, EC2 and the revertible strains of EC2 lacked the *can* gene. Multi-locus sequence typing showed that all strains belonged to ST457 and nucleotide similarity analysis indicated that they had high homology. In conclusion, we report rarely described co-isolated forms of three phenotypes of *E. coli* that included acapnophilic SCV in a patient with bacteremia. The capnophilic SCV strain had atypical morphology and biochemical characteristics with the absent of *can* gene. Based on our findings, we have discussed the laboratory identification, characterization, mechanisms, and clinical treatment of capnophilic SCV strains.

Introduction

Bacteria are capable of adapting to environmental pressures through changes in their phenotype and genetics. Small colony variants (SCVs) constitute a naturally occurring sub-population of bacteria with atypical colony morphology and unusual biochemical characteristics. The most conspicuous feature of SCVs is the small size of the colonies compared with wild-type bacteria,the slow growth rate, and low susceptibility to antibiotics, which pose a challenge for clinical identification and treatment (ProctorRA et al. 2006). At present, SCVs have been identified in several genera of bacteria (Swingle EL 1935; Bryan LE et al. 1981; Mowjood M et al. 1979). The greatest amount of information about SCVs has been obtained from *Staphylococcus aureus*, while there have been very few reports about SCVs in clinical specimens of *Escherichia coli* (Roggenkamp A et al. 1998; Tappe D et al. 2006; Negishi T et al. 2018). *E. coli* SCVs were first described on 1931 (Hadley P et al. 1931), and there were subsequent studies on the phenotypic characteristics, clinical infection, and molecular mechanism of *E.coli* SCVs.

Most SCVs that have been recovered so far are deficient in electron transport and thymidine biosynthesis, and these phenotypes can be reversed by supplementation with menadione, hemin, or thymidine (ProctorRA et al. 2006). *E.coli* SCVs with *hemB*, *hemD*, *hemL* or *lipA* mutations have been used for functional characterization of changes in growth, metabolism, antimicrobial susceptibility, and persistence (Ramiro RS et al. 2016; Santos V et al. 2016). However, capnophilic *E. coli* have rarely been reported. The first case of bacteremia by capnophilic *E. coli* showed genetic loss of the *YadF* gene (Sahuquillo-Arce JM et al. 2017), and in 2018, co-expression of *E.coli* SCVs and *E. coli* with capnophilic phenotypes was reported in a patient with urinary tract infection (Park YJ et al. 2018). However, the genetic and biochemical basis of *E.coli* SCVs and capnophilic *E. coli* emergence in the in vivo context is not well understood.

With regard to their clinical characteristics, SCVs are better able to persist in cells and are less susceptible to antibiotics than their wild-type species (ProctorRA et al. 2016). These characteristics make their clinical identification difficult and lead to persistent or recurrent infection on emergence from the protective environment of the host cell. From the clinical microbiology perspective, it has been difficult to identify and assess the antimicrobial susceptibility of SCVs due to a lack of established standards or guidelines. Therefore, the incidence of SCVs in clinical specimens may not reflect their true prevalence, and some patients may suffer from persistent infection because of SCVs. Therefore, it is important to focus on the monitoring and detection of SCVs with comprehensive studies on the phenotypic and genetic characterization of *E. coli* SCVs.

In this study, we report three different phenotypic strains of *E. coli*, including a wild-type, a capnophilic, and a capnophilic SCV form, that were co-isolated from a patient with bacteremia in China. We examined the phenotypic features, laboratory diagnosis, and antimicrobial sensitivity of the three strains. In addition, whole genome sequencing (WGS) was performed to determine the causal genetic mechanisms that distinguish the capnophilic *E. coli* and capnophilic SCV from the wild-type strain. The results provide important evidence for the identification, mechanisms, and clinical treatment of capnophilic SCVs.

Materials And Methods

Clinical features of the patient

A 64-year-old woman with kidney stones and hydronephrosis was admitted to Nanjing Drum Tower Hospital on April 22, 2018. After percutaneous nephrolithotomy on April 24, the patient had high fever with a body temperature of 40.7°C and signs of septic shock. Her white blood cell count was 15.8×10^9/L; neutrophil percentage, 97.1%; C-reactive protein level, 65.9 mg/L; and procalcitonin, 4.94 ng/mL.

Bacterial culture and isolation

A urine sample and two sets of blood samples were cultured for bacterial identification. Gram-negative bacilli were identified on microscopic examination after 18-h culture of the blood samples in aerobic and anaerobic bottles. The samples were also cultured on a sheep blood agar plate (BAP) at 35°C in a 5% CO_2 atmosphere overnight, and a slow-growing, needle-tip-like small colony was identified on the BAP. We named the three strains of Gram-negative colonies isolated from blood culture as EC1, EC2, and EC3. EC1 and the needle-tip-like EC2 were isolated from the same aerobic culture bottle, while EC3 was isolated from the anaerobic culture bottle. The urine sample was cultured on sheep BAP at 35°C in a 5% CO_2 atmosphere. After 24 h of incubation, >10,0000 CFU/ml of Gram- negative colonies were detected on the BAP. The strains identified in the blood and urine samples were isolated for further characterization.

Treatment and outcome

The patient was transferred to the ICU for replenishment of blood volume by treatment with a vascular active medicine, and also received oxygen inhalation treatment. For anti-infection therapy, the patient was treated with imipenem (500 mg, Q8H) for one week. Based on elevated blood creatinine level (136 mmol/L) and oliguria, acute renal injury was diagnosed and the patient received renal replacement therapy. The patient recovered well and was discharged on May 9. The study protocol was approved by the Ethics Committee of Nanjing Drum Tower Hospital.

Colony morphology analysis and Gram staining

The morphological characteristics of strains EC1, EC2, and EC3 were compared in three different culture conditions: aerobic, 5% CO_2, and anaerobic. We also observed the morphological characteristics of the strains at three time points: after 24, 48, and 72 h of culture. The EC1, EC2, and EC3 bacterial cells were also analyzed by Gram staining. *E. coli* ATCC 25922 was used as the control strain.

Identification of strains

The three strains, EC1, EC2, and EC3, were identified using the VITEK 2 system (bioMérieux, France) and the API 32E system (bioMérieux, France) according to the manufacturer's instructions. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (micro Typer MS) was used to confirm the identification. The colonies isolated from urine were identified by the VITEK 2 system. The biochemical characteristics of the strains were examined with the oxidation fermentation experiment and oxidase test.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the VITEK 2 system with the GN13 card and the disk diffusion method (Oxoid Lid, UK). For the disk diffusion method, a 0.5 McFarland suspension of each of the three strains was prepared and spread on Mueller Hinton (MH) agar and cultured in a 5% CO_2 environment for 16–18 h with the following antimicrobials: ampicillin, ampicillin-sulbactam, amikacin, ceftazidime, cefotaxime, cefuroxime, cefoxitin, imipenem, piperacillin-tazobactam, cefepime, levofloxacin, and sulfamethoxazole-trimethoprim. The minimum inhibitory concentration (MIC) and disk diffusion method results were determined based on the criteria for testing *Enterobacter*according to CLSI M100 (Clinical and Laboratory Standards Institute 2018).

Auxotrophy testing

Auxotrophy tests for thymidine, hemin, thiamine, and menadione were performed as described previously (Wellinghausen N et al. 2009). The final concentration of the four supplements was 10 μg/ml in MH agar. EC1, EC2, EC3, and ATCC25922 were inoculated on the MH agar and incubated at 35°C in 5% CO_2 for 48 h.

Preparation of genomic DNA and WGS analysis

Genomic DNA of the three strains was extracted from a culture grown overnight in Luria-Bertanimedium at 35°C under agitation (220 rpm) using the QIAamp DNA minikit (Qiagen, Hilden, Germany). Library preparations were constructed following the manufacturer's protocol (Illumina TruSeq DNA Nano Library Prep Kit) and sequenced using the IlluminaHiseq PE150 platform to generate 450-bp paired-end reads.Trimmed sequencing reads were subjected to *de novo* assemblywith SPAdes pipeline version 3.9.0 (Bankevich A et al. 2012) under the default settings. Multi-locus sequence typing (MLST) was performed according to a previously published method (Larsen MV et al. 2012). WGS was performed to identify genes that contained previously reported causative mutations for the *E. coli* SCV phenotypes and the nucleotide similarity was analyzed by CLC Genomics Workbench 21.0.3. The genome sequences of EC1, EC2, and EC3 have been deposited in GenBank under accession numbers QVHR00000000, QVHQ00000000, and QVHS00000000, respectively.

PCR analysis

Extracted DNA was amplified by PCR under standard conditions as described previously (Sahuquillo-Arce JM et al. 2017) with the following primers for amplification of the 510-bp fragment of the *can* gene (which is not present in capnophilic strains): *can*F (5'-

TTGGTGGCGGTAACATCCAG-3') and *can*R (5'-GAAACTGGCACAAGCGC AAA-3'). The following PCR protocol was used: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension of 7 min at 72°C. The amplification products were separated by electrophoresis on an agarose gel with 2% ethidium bromide staining, and the size of the amplicons was determined based on their homology with a molecular weight marker (PCR marker; Promega Corporation, Madison, WI, USA) DL2000.

Results

Colony and cell morphologies of the three co-isolated strains

After 48 h of incubation on BAP at 35°C in a 5% CO_2 atmosphere, the EC1 and EC3 colonies appeared sleek and greyish, but the EC1 colony appeared slightly drier than the EC3 colony. In contrast, the EC2 colonies had a needle-tip-like appearance and were dry and not easy to pick from the BAP surface (Fig. 1A). Gram staining showed that EC1 and EC2 were heterogeneous, swollen, and elongated, and most of the EC3 cells were of the same size as *E. coli* ATCC 25922 cells (Fig. 1B).

Growth characteristics of strains under different conditions

We compared the morphology of the three colonies in different culture environments (aerobic, 5% CO_2, and anaerobic) and at different time points (24, 48, and 72 h) (Fig. 2). EC1 and EC2 failed to grow under aerobic conditions in the absence of CO_2 and EC3 showed growth in this condition. Under the 5% CO_2 condition, EC2 colonies showed slow growth and tightly adhered to the BAP. All three isolates grew in the anaerobic environment, but appeared paler than they did in the CO_2 environment (Fig. 2). Notably, EC2 exhibited the ability to revert to the large-form EC1 phenotype in the presence of CO_2, and did not demonstrate growth defects under anaerobic conditions.

Differences in biochemical characteristics

Biochemical identification with the VITEK 2 system and API 32E system identified all three colonies as *E. coli*, and this was confirmed by MALDI-TOF MS. In contrast to EC3 (which was identified as a wild-type strain), EC1 and EC2 did not ferment glucose or express tyrosine arylaminase. EC2 was positive for oxidase and the others were negative (Table 1). The strain isolated from the urine sample was identified as *E. coli* and had the same biochemical characteristics as the wild-type EC3 strain.

Table 1
Differences in biochemical reactions of three strains of *E.coli*

biochemical reaction	EC1	EC2	EC3
BGAL	+	+	+
H2S	-	-	-
dGLU	+	+	+
OFF	-	-	+
dMAL	+	-	+
dMAN	+	+	+
TyrA	-	-	+
URE	-	-	-
SAC	+	-	+
dTAG	-	-	+
MNT	-	-	-
PHOS	-	-	+
ODC	-	-	-
O129R	-	-	+
OX	-	+	-
catalase	+	+	+
IND	+	+	+

Antimicrobial susceptibility results

The capnophilic EC1 and EC2 strains could not be cultured in ambient air with the VITEK 2 system, and this was indicative of terminated. The disk diffusion method under 5% CO_2 conditions showed that EC1, EC2, and EC3 were resistant to ampicillin, ampicillin-sulbactam, amikacin, levofloxacin, and sulfamethoxazole-trimethoprim, and they were susceptible to cefoxitin and imipenem (Table 2). Additionally, EC1 and EC2 were resistant to ceftazidime and cefepime, while EC3 exhibited intermediate resistance to ceftazidime and cefepime. Further, EC2 and EC3 were susceptible to piperacillin-tazobactam, while EC1 showed intermediate susceptibility. The *E. coli* strain isolated from the urine sample had similar antimicrobial susceptibility features as EC3.

Table 2
Antimicrobial susceptibility results of three strains of *E.coli*

Antimicrobial agent	**disk diffusion method (5% CO_2)**			**VITEK 2 system GN13 card**		
	EC1	EC2	EC3	EC1	EC2	EC3(MIC)
Ampicillin	6R	6R	6R	Terminated	Terminated	>=32R
Ampicillin-sulbactam	8R	6R	8R			>=32R
Amikacin	6R	6R	6R			>=64R
Ceftazidime	17R	15R	18I			8I
Cefotaxime	6R	6R	6R			
Cefuroxime	6R	6R	6R			
Cefoxitin	20S	25S	20S			<=4S
Imipenem	27S	30S	26S			<=1S
Piperacillin-tazobactam	20I	26S	22S			<=4S
Cefepime	6R	6R	15I			4I
Levofloxacin	6R	6R	6R			>=8R
SMZ-TMP	6R	6R	6R			>=320R

Auxotrophic features of EC1, EC2, and EC3

After culturing for 48 h, the growth of EC2 on MH agar containing thymidine, hemin, thiamine, and menadione was not enhanced. Under aerobic conditions, EC1 and EC2 were unable to grow in the presence of thymidine, hemin, thiamine, and menadione except EC3 (Fig. 3).

WGS analysis

The results of WGS of the three isolated *E. coli* strains were highly consistent (Table 3). A set of 6 virulence genes and 13 resistance genes were identified in each strain with the VirulenceFinder (2.0) and ResFinder (3.2) servers of the Center for Genome Epidemiology website. MLST results showed that all three strains belong to ST457 (MLST 2.0, http://www.genomicepidemiology.org). Nucleotide similarity analysis indicated that the three strains had high homology (> 99%). With regard to single nucleotide polymorphism (SNP), 4 high-quality SNPs were detected between EC1 and EC2, while 34 high-quality SNPs were detected between EC1 and EC3. However, no genetic mutational variations associated with SCVs (in the *hemB*, *thyA*, and *lipA* genes) were observed in the three strains. The *can* gene, which is absent in capnophilic *E. coli* strains, was not present in EC1 and EC2 but expressed in EC3. The PCR results confirmed that the *can* gene was present in EC3 and ATCC25922 *E. coli* but absent in EC1, EC2 and the revertible isolate of EC2.

Table 3
Information of whole genome sequencing of three strains of *E.coli*

strains	contigs	genes	Proteins	rRNA	tRNA	ncRNA	bases	N50	MLST	Plasmids	Virulence Genes	Resistance Genes
EC1	125	5326	4984	12	82	7	5130567	242130	ST457	IncX1 p0111	eilA lpfA air iss gad	aph(3')-IIa aadA2 rmtB strB strA fosA sul2 sul1 dfrA12 tet(A) floR blaCTX-M-27 blaTEM-1B
EC2	124	5342	4999	12	83	7	5146176	241168				
EC3	120	5343	4999	12	82	7	5145687	241168				

Discussion

SCVs are usually associated with chronic persistent infection. There are very few reports about clinical specimens of *E. coli* SCVs that cause urinary tract infection, prosthesis-related infection, and bacteremia. From 1977 to 1978, Borderon et al 1978 observed 16 small colony mutants from 745 strains of *E. coli* isolated from urine that caused pyuria and persistent urinary tract infection. In 2005, a case of febrile bacteremia due to an *E. coli* SCV was reported, in which the patient also experienced a long-term urinary tract infection (Tappe D et al. 2006). Additionally, two studies reported that *E. coli* SCVs were responsible for infection of the prosthesis joint, which is a serious complication that occurs after joint replacement (Roggenkamp A et al. 1998; Sendi P et al. 2010). In 2018, co-existing *E. coli* SCVs and capnophilic phenotypes isolated from a patient with urinary tract infection were first reported (Park YJ et al. 2018). However, we believe that the actual clinical prevalence of infections caused by *E. coli* SCVs is far greater than the reported incidence so far. In the present study, we describe three different phenotypic strains of *E. coli*, including a wild-type strain, a capnophilic strain, and a capnophilic SCV, that rarely co-existed in a patient with kidney stone-associated infection and bacteremia. The capnophilic strain and capnophilic SCV that were isolated from her blood sample had different growth and biochemical features, while WGS analysis indicated that the three different phenotype strains were highly consistent.

Mutations of the gene encoding the key enzyme in the electron transfer system and thymidylate synthase are responsible for the emergence of SCVs. In addition, biofilm and antibiotic pressure also promote such variations. In 1998, Roggenkamp et al. 1998 reported the small colony mutant *hemB E. coli* in a case of artificial hip joint infection for the first time. Further, according to a recent report, a novel SNP in *hemA* of a clinical isolate of *E. coli* was responsible for an SCV phenotype (Hubbard ATM et al. 2021). With regard to thymidine-dependent SCVs of *E. coli*, mutation in the thyA gene (c.62G > A) was found to cause morphological abnormalities in the colonies and thymidine auxotrophy (Negishi T et al. 2018). Recent studies also showed that mutation in the lipoic acid synthase gene (*lipA*) resulted in SCV phenotypes and slow growth of *E. coli* bw25113. Additionally, expression of the biofilm genes *wcaC* and *wcaK* in the *lipA* mutant strain was also higher than that in the wild-type strain (Santos V et al. 2016). In our report, the SCV EC2 did not exhibit thymidine auxotrophy or electron transport defects. Further, EC2 had the ability to revert to the large-form EC1 phenotype in the presence of CO_2, and the reverted colonies also exhibited a capnophilic phenotype. This indicates a potential in vivo survival mechanism of *E. coli* SCVs. Similar findings have been reported for Enterobactericeae by Alexander et al. 2021. No genetic mutations associated with the *E. coli* SCV phenotype were detected in the *hemB*, *thyA*, and *lipA* genes in the three strains. Capnophilic *E. coli* have rarely been reported (Matsumoto T et al. 2020; Lu W et al. 2012; Tena D et al. 2018), so our strains make an important contribution to the database of capnophilic *E. Coli*. We did not detect the *can* gene in EC1 and EC2, and this finding is consistent with previous reports on capnophilic *E. coli* (Sahuquillo-Arce JM et al. 2017; Park YJ et al. 2018). Carbonic anhydrase activity is supplied by the product of *can* which is essential for aerobic growth of *E.coil*, and the impaired carbonic anhydrase function can cause a carbon dioxide-dependent SCV phenotype in *E.coil* (Matsumoto T et al. 2020). The nucleotide similarity analysis indicated that the three strains had high homology, and the

data of WGS showed highly consistent in virulence and resistance genes. Further research on the transcription level and protein level of these strains could shed light on the mechanism of the emergence of capnophilic SCVs.

The identification and treatment of SCV infections have posed a challenge to microbiologists and clinicians. In addition to their atypical colony morphology, they also have a deficiency in biochemical reactions. Therefore, it is difficult to identify them based on their biochemical profile. Genetic profiling with methods such as 16S rRNA sequencing, MALDI-TOF MS, and WGS are of great value in the identification and characterization of SCVs. In order to reduce the possibility of missing CO_2-deficient SCVs, the bacterial samples should be cultured in a 5% CO_2 environment for a prolonged time. Due to the slow growth and low metabolic rate of SCVs, conventional antimicrobial susceptibility tests, including the disk diffusion method, E-test, micro-dilution tests, and automated susceptibility testing systems, may result in errors (ProctorRA et al. 2006). The antimicrobial susceptibility results in our report were not significantly different, but this is probably due to the slow growth of SCVs. Therefore, for antimicrobial susceptibility testing of SCVs, it is necessary to ensure that the bacteria are cultured for a sufficient amount of time on the plate or in the liquid according to colony growth, and the MIC values must be carefully evaluated. The most suitable methods for antimicrobial susceptibility testing of *E. coli* SCVs need to be further explored. For antibiotic therapy in the clinical setting, all the co-existing strains and SCVs must be considered. Secondly, it is important to avoid certain antibacterial drugs, such as aminoglycosides, based on the auxotrophic features of SCVs, as some findings have shown that SCVs of *E. coli* are resistant to aminoglycoside antibiotics (Lewis LA et al. 1991). In the present case, there was no significant difference in the drug sensitivity of the three strains of *E. coli*, but they were all resistant to aminoglycosides. The patient was treated with imipenem and was discharged in good health.

Conclusions

We report three different phenotypic strains of *E. coli*, including a wild-type strain, a capnophilic strain, and a capnophilic SCV, that were co-isolated from a patient with bacteremia. The three phenotypes of *E. coli* showed high homology in nucleotide level, and capnophilic SCV strain had atypical morphology and biochemical characteristics with the absent of *can* gene. The phenotypic changes, production mechanism, laboratory identification, and clinical treatment of SCVs were discussed. More research is needed on the mechanism by which *E. coli* SCVs emerge, their reverse mutations, new gene mutation sites, targeted laboratory detection methods, and antimicrobial susceptibilities. These SCVs can also shed more light on bacterial metabolism, signaling, and virulence. Importantly, more research on these SCVs could improve their diagnosis and monitoring, as well as the treatment and outcome of persistent infection caused by SCVs.

Abbreviations

SCV: Small colony variants; *E.coli: Escherichia coli*; WGS: whole genome sequencing; BAP: blood agar plate ; MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MH: Mueller Hinton; MIC: minimum inhibitory concentration; MLST: multi-locus sequence typing; SNP: single nucleotide polymorphism.

Declarations

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Conflicts of interest/Competing interests

All authors declare that they have not conflict of interest.

Availability of data and material

Whole genome sequencing data are available in GenBank with the accession number QVHR00000000, QVHQ00000000 and QVHS00000000, respectively.

Code availability

Not applicable

Authors' contributions

GS and ZW were responsible for the patient’s management, data collection and drafted the manuscript. ZZ and XX performed the culture and molecular experiment. ZH and ZH were responsible for the identification and antimicrobial susceptibility test. ZY was responsible for editing

the figures. CX performed WGS and analyzed the data. SH was responsible for interpretation of data and supervised this manuscript. All authors have read and approved the final manuscript.

Ethics approval

Ethical approval for this study was granted by the ethics committee of the Nanjing Drum Tower Hospital

Consent to participate

Written informed consent was obtained from the patient to participate this study.

Consent for publication

Written informed consent was obtained from the patient for publication of this paper.

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Figures

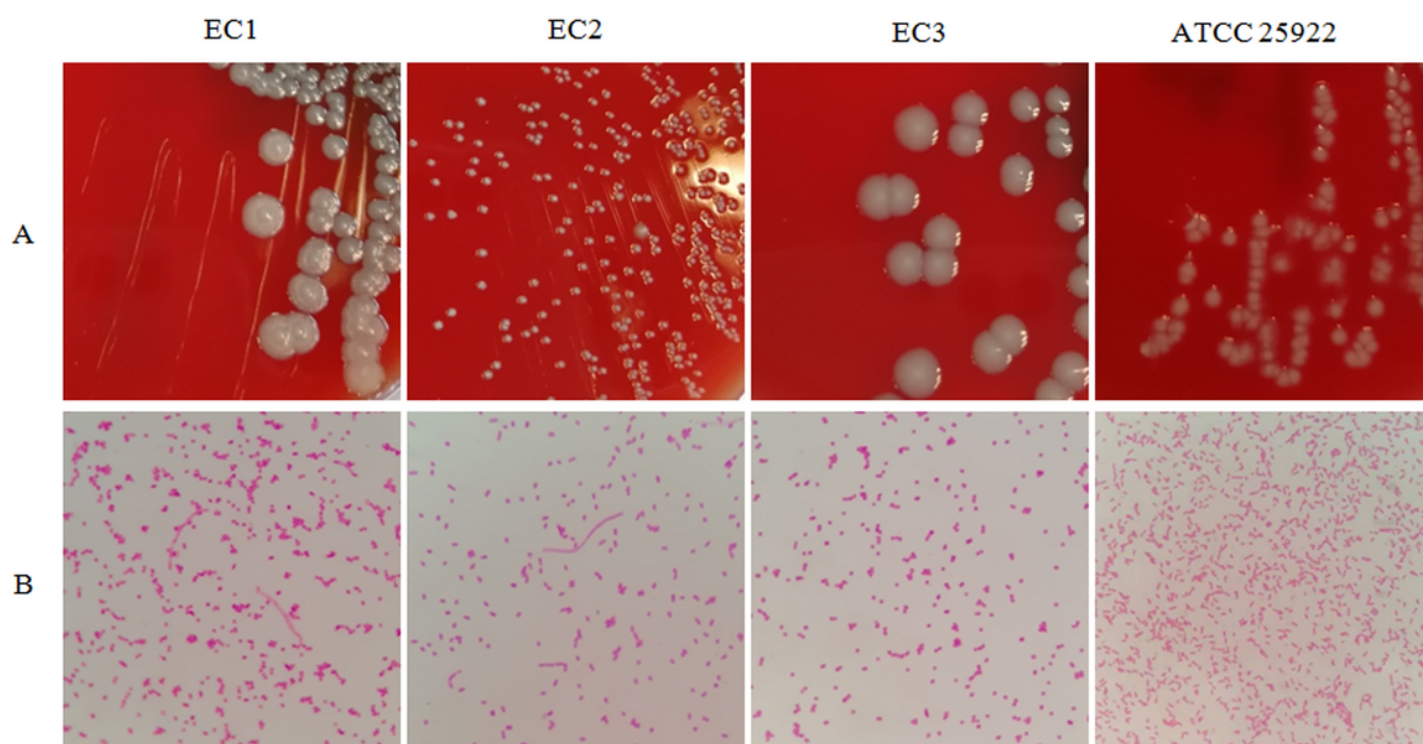

Figure 1

Colonies and cell morphologies of three co-isolated strains from blood culture of patient. A：Colony morphology of EC1, EC2, EC3 and ATCC25922 after cultured on BAP in 5% CO2 for 48h. B: Bacterial cell morphologies of EC1, EC2, EC3 and ATCC25922. Gram staining, visualized at 1000 magnification.

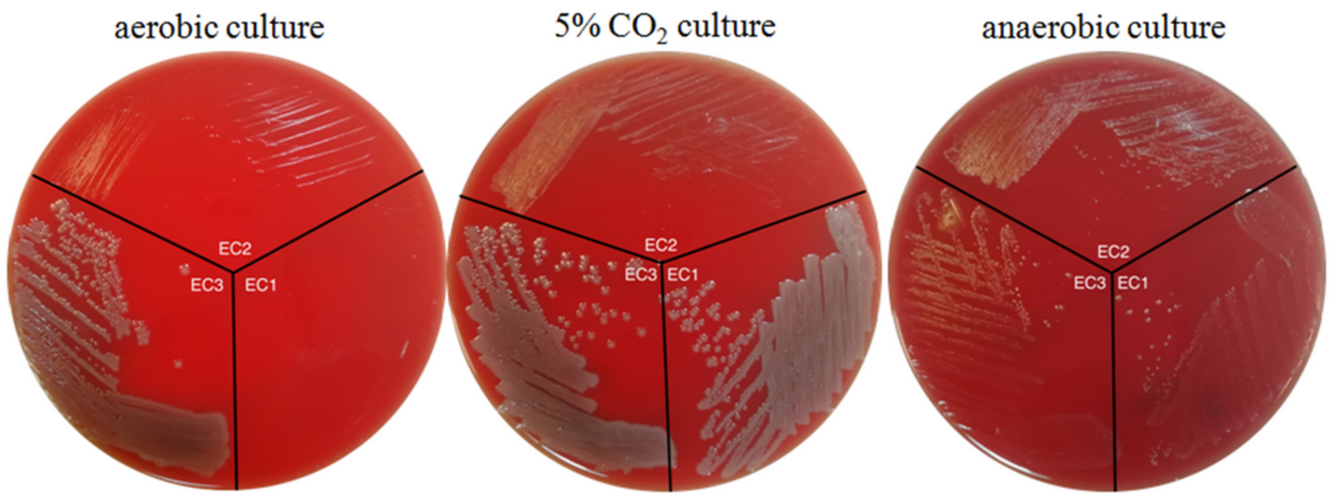

Figure 2

Colony morphologies of three strains in different culture condition. Colony features of EC1, EC2 and EC3 E.coli were showed after culturing on BAP in aerobic, 5% CO2 and anaerobic environment for 24 hours.

Figure 3

Auxotrophy testing of three E.coli strains and ATCC25922 with thiamine, menadione, hemin and thymidine. The strains of EC1, EC2, EC3 and ATCC25922 were inoculated on the MH agar with four supplement which the final concentration is 10 μg/ml, followed by incubation at 35 °Cin 5% CO_2 for 48h.

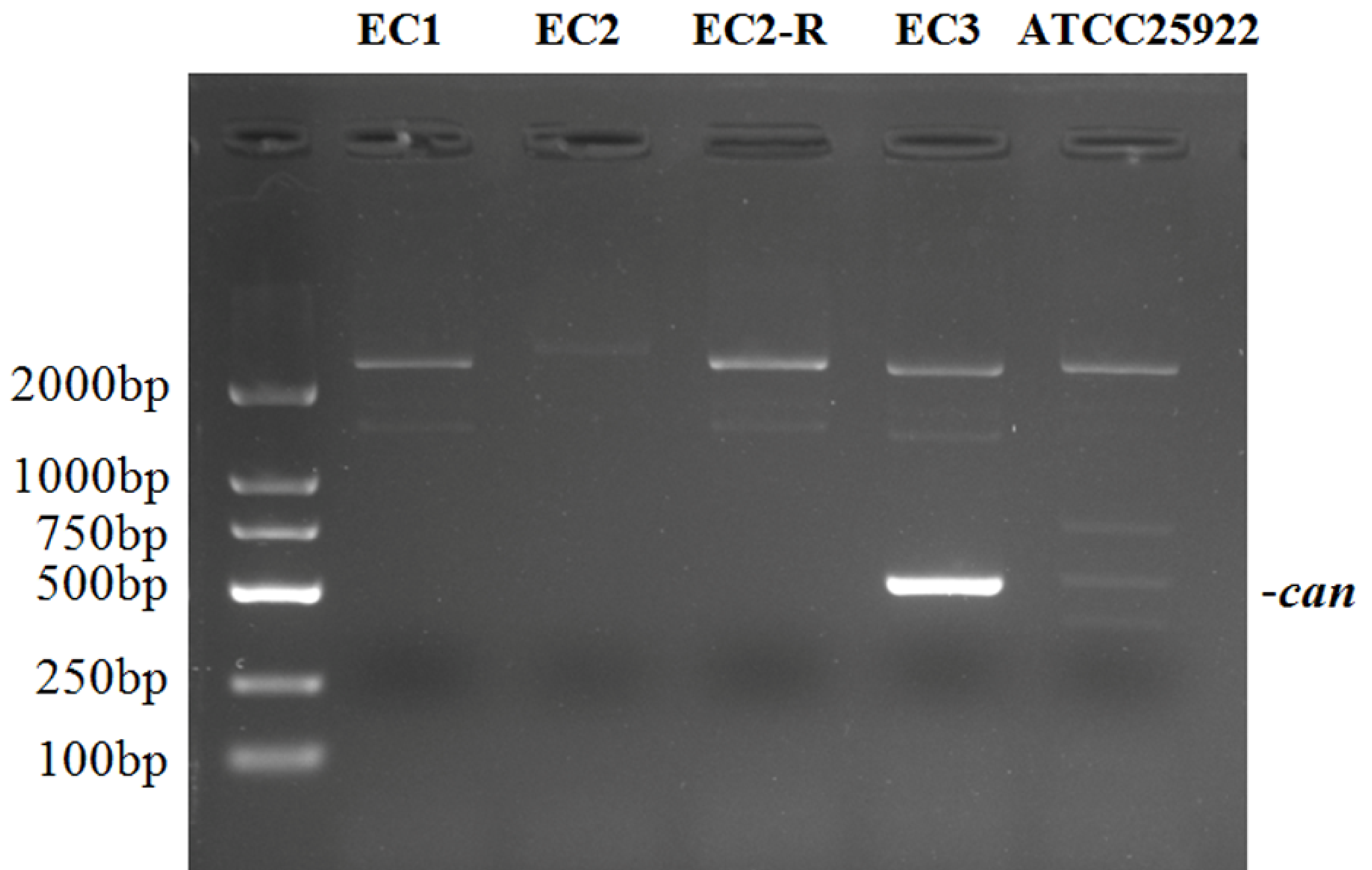

Figure 4

The PCR results of can gene in the strains including the reverted isolate and ATCC25922. EC2-R: the reverted isolate of EC2.