Genotypic study of *Citrobacter koseri*, an emergent platelet contaminant since 2012 in France

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BACKGROUND: Transfusion-transmitted bacterial infection is a rare occurrence but the most feared complication in transfusion practices. Between 2012 and 2017, five cases of platelet concentrates (PCs) contaminated with the bacterial pathogen *Citrobacter koseri* (PC-*Ck*) have been reported in France, with two leading to the death of the recipients. We tested the possibilities of the emergence of a PC-specific clone of *C. koseri* (*Ck*) and of specific bacterial genes associated with PC contamination.

STUDY DESIGN AND METHODS: The phylogenetic network, based on a homemade *Ck* core genome scheme, inferred from the genomes of 20 worldwide *Ck* isolates unrelated to PC contamination taken as controls (U-*Ck*) and the genomes of the five PC-*Ck*, explored the clonal relationship between the genomes and evaluated the distribution of PC-*Ck* throughout the species. Along with this core genome multilocus sequence typing approach, a *Ck* pan genome has been used to seek genes specific to PC-*Ck* isolates.

RESULTS: Our genomic approach suggested that the population of *C. koseri* is nonclonal, although it also identified a cluster containing three PC-*Ck* and eight U-*Ck*. Indeed, the PC-*Ck* did not share any specific genes.

CONCLUSION: The elevated incidence of PCs contaminated by *C. koseri* in France between 2012 and 2017 was not due to the dissemination of a clone. The determinants of the recent outbreaks of PC contamination with *C. koseri* are still unknown.

Transfusion-transmitted bacterial infection (TTBI) is defined as clinical features of bacteremia or sepsis during or after transfusion of contaminated blood products, with a relevant blood culture in the recipient.[1] Although TTBIs are rare events, they are life-threatening complications and one of the most feared in transfusion.

Most bacteria contaminating blood products are part of normal skin flora and may be introduced when the needle crosses the skin barrier.[2] More rarely, bacteria may be also drawn with blood from an asymptomatic bacteriemic donor (e.g., after dental procedure, due to a gastrointestinal lesion, or a donor with an asymptomatic urinary tract or digestive infection).[2] Finally, bacterial contamination may occur from the environment during platelet storage and transports through defects in the storage container.[3]

ABBREVIATIONS: AS = additive solution; cgMLST = core genome multilocus sequence typing; CRB-FMB = Centre de Ressources Biologiques–Filière Microbiologique de Besançon; NCBI = National Center for Biotechnology Information; PCs = platelet concentrates; PC-*Ck* = platelet concentrate contaminated with *Citrobacter koseri*; PC-P = Platelet concentrate in plasma; TTBI = transfusion-transmitted bacterial infection; U-*Ck* = *Citrobacter koseri* isolates unrelated to PC contamination.

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TABLE 1. Collection of five *C. koseri* isolates involved in TTBI or contamination of PCs between 2012 and 2017 in France. Investigation of contaminated PCs or TTBI events by the French hemovigilance organization quantifies imputability and severity (from 1 to 4) with high levels corresponding to strong suspicion

Isolate	Year of isolation	City of origin	Blood product*	Level of imputability	Level of severity	Clinical context†	Isolation site from donor	NCBI biosample reference
MTP	2012	Montpellier	APC-AS	2	2	10-year old female, post chemotherapy febrile neutropenia	…	SAMN11265047
PAR	2015	Paris	APC-AS	3	4	8-year-old female, congenital dyskeratosis, severe aplastic anemia	Nose	SAMN11265045
LIL	2015	Lille	WBDP‡	2	1	…§	…	SAMN11265046
BES	2017	Besançon	WBDP-AS	2-3	3-4	60-year-old female, leukemia	Armpit	SAMN11265048
NAN	2017	Nantes	WBDP-AS	2-3	1	…§	…	SAMN11265049

* APC = apheresis platelet concentrate; AS = additive solution; WBDP = whole-blood-derived platelets.
† For details on the isolates MTP, PAR, and BES, see References 9,10,14, respectively.
‡ Platelet storage medium unknown.
§ The origin of the contamination by the isolates LIL and NAN, retrieved from PCs discarded before administration to patient because of invalid organoleptic quality control, was not further investigated.

To lower these risks of bacterial contamination of blood products, measures have been implemented in France and in many countries, including thorough routine donation screening, skin asepsis, and donation testing protocols.[2] Moreover, from the time of donation and throughout its shelf life, each blood donation is monitored with multiple quality controls. In France, no systematic bacterial culture screening of platelet components is done, but the products suspected of contamination are quarantined and/or discarded. Since 1994, to identify opportunities for improvement, all cases of definite or probable TTBI are reported to the French hemovigilance organization, which publishes annual reports.[4–6] All the precautions implemented since 1994 dramatically reduced the number of TTBIs in France over the past decades. However, the risk of death due to TTBI has remained quite stable since 1994, notably with platelets concentrates (PCs).[6] Indeed, among all the blood products, PCs represent the most important infection risk of bacterial contamination, as their storage at room temperature allows growth of contaminating bacteria to lethal concentrations.[2] Subsequently, to reduce the bacterial risk further, French authorities decided in 2017 to implement pathogen inactivation for all PCs (INTERCEPT, Cerus BV) nationwide.[7,8] Besides, almost all the PCs produced in France are prepared in platelet-additive solution (PAS) at the moment.

Despite all these precautions and before the implementation of pathogen inactivation technology, the review of French hemovigilance organization annual reports revealed

TABLE 2. Collection of 20 isolates of *Citrobacter koseri* used as control for genotypic characterization of isolates contaminating platelet concentrates

Isolate	Geographic origin	Isolation site	Collection date	NCBI biosample reference
U-*Ck* isolates used as phenotypic controls and for phylogenetic analysis				
D6H	Besançon (France)	Blood	2017	SAMN11265053
D7B	Besançon (France)	Feces	2017	SAMN11265054
D8I	Besançon (France)	Bone	2015	SAMN11265051
E1H	Besançon (France)	Blood	2016	SAMN11265052
E8B	Besançon (France)	Feces	2012	SAMN11265050
Genomic data used for phylogenetic analysis				
FDAARGOS_393	Maryland (United States)	Blood	2005	SAMN07312437
FDAARGOS_287	Maryland (United States)	Urine	2014	SAMN06173300
ATCC BAA-895	Missouri (United States)	Cerebrospinal fluid	1983	SAMN02603912
B1B	Kuala Lumpur (Malaysia)	Tooth	2013	SAMN05371063
FDAARGOS_164	Maryland (United States)	Urine	2014	SAMN03996311
FDAARGOS_86	Maryland (United States)	Urine	2013	SAMN02934540
DNF00568	Missouri (United States)	Vagina	2015*	SAMN03842473
ERS712579	Hinxton (United Kingdom)	Respiratory tract	2015*	SAMEA3359025
GED7778C	Missouri (United States)	Vagina	2015*	SAMN03842474
PSS_7778B	Missouri (United States)	Urine	2015*	SAMN03842475
0123A_53_520	Pittsburg (United States)	Feces	2014	SAMN05509313
SCCK020020	Sichuan (China)	Human sample	2016	SAMN08027249
DRS013905	Gifu (Japan)	Human sample	2014*	SAMD00012414
DRS013830	Gifu (Japan)	Human sample	2014*	SAMD00016622
ERS485760	Marseille (France)	Rat	2014*	SAMEA2612307

* Submission date to the National Center for Biotechnology Information, when collection date was unavailable.

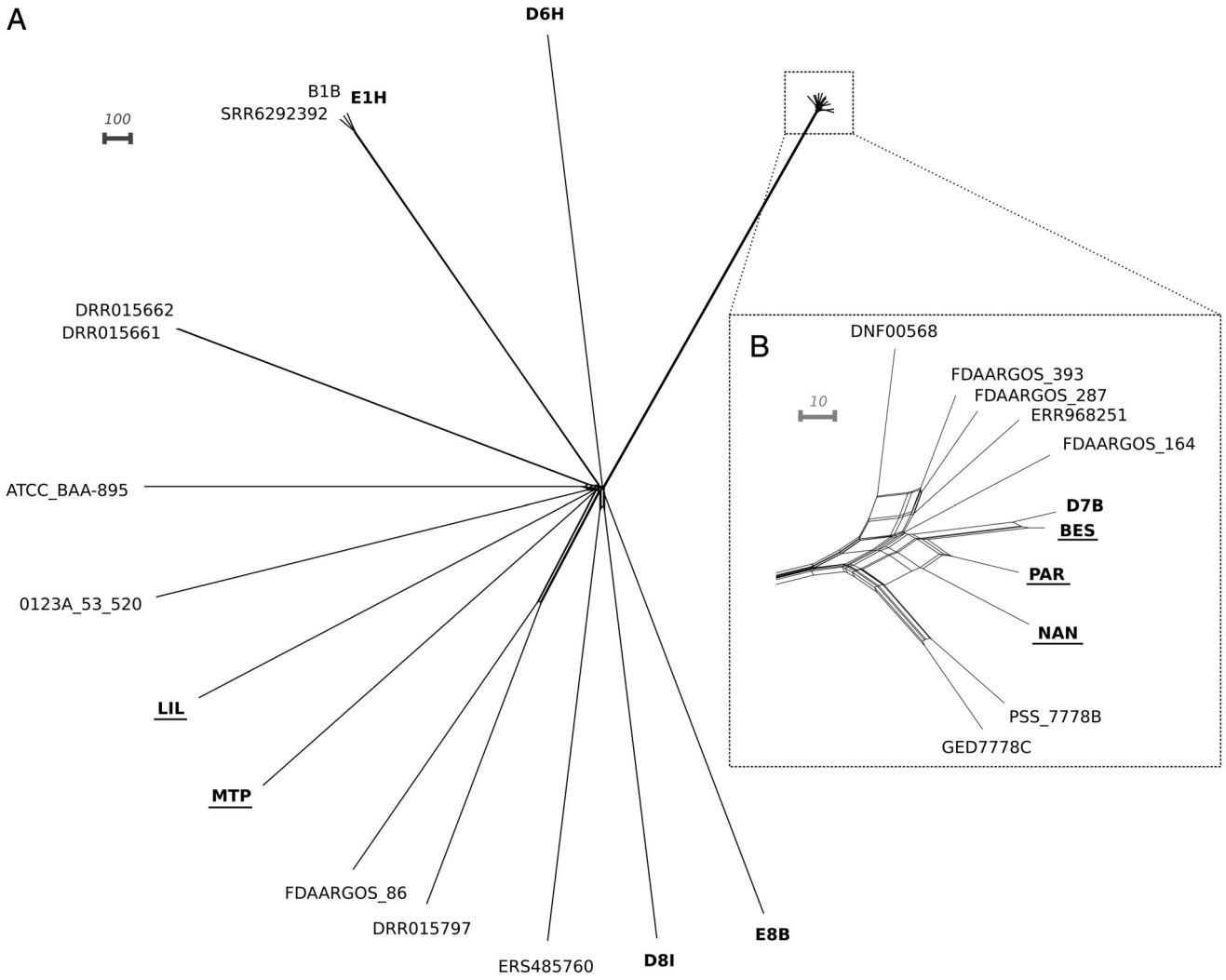

Fig. 1. Phylogenetic network of the genomes of 25 *C. koseri* isolates, including five isolates contaminating PCs (underlined bold characters). (A) The genomic comparison included five isolates (bold characters) collected at the University Hospital of Besançon (France) and 15 genomes available in NCBI database. We identified 4950 genes shared by ≥96% of the selected genomes (≥24/25 genomes). The MLST analysis was based on this core genome. The dotted circle indicates Cluster A. (B) Close-up of Cluster A.

an outbreak involving the gram-negative bacillus *Citrobacter koseri*. Indeed, this species has been involved in 5 of 19 cases of PC contamination reported between 2012 and 2017, while it was never involved in a TTBI event before 2012 (Pascal Morel, personal data).[6] Two contaminations led to the death of the two recipients.[9,10] *C. koseri* is an Enterobacteriaceae that gets its species name from its ability to use citrate as the sole carbon source. *C. koseri* is commonly found in water, sewage, soil, and food. It occasionally colonizes the gastrointestinal tract of mammals and the human skin and may also cause serious infections in immunocompromised hosts.[11]

In this study, the question of a common source of contamination from environment (e.g., on platelet agitators or by a common source such as blood bags or apheresis kit) or the selection of a well-adapted *C. koseri* clone, belonging to donors' microbiota and prone to proliferate in PCs, was investigated.

For this purpose, we evaluated the relationships between PC-associated isolates through a core genome multilocus sequence typing (cgMLST) approach, which is a high-discriminatory typing method based on the gene-by-gene comparison, and sought genes specifically borne by PC-associated *C. koseri*. In preliminary tests, we also compared the growth of PC-*Ck* and U-*Ck* isolates in PCs suspended in either PAS or plasma.

MATERIAL AND METHODS

Collection of *C. koseri* isolates

The Centre de Ressources Biologiques–Filière Microbiologique de Besançon (CRB-FMB, Biobanque BB-0033-00090) provided the five *C. koseri* transfusion-related isolates (PC-*Ck*) as well as

five transfusion nonrelated isolates of *C. koseri* (U-*Ck*) retrieved from human samples in the University Hospital of Besançon between 2012 and 2017. Table 1 details the characteristics of PC-*Ck* since 2012, including the clinical context of isolation. Table 2 shows compiled information about the 20 U-*Ck* (including the five above-mentioned U-*Ck*) analyzed with the five PC-*Ck* to define the cgMLST scheme. Five U-*Ck* provided by the CRB-FMB have been used as well as all the publicly available genome sequences from the National Center for Biotechnology Information (NCBI) database. Eleven assembled genomes and four sets of raw reads were available at the time of this study (March 2018).

DNA extraction and raw reads assembly

Bacterial DNA of PC-*Ck* and U-*Ck* provided by CRB-FMB was extracted with a DNA purification kit (QIAamp DNA Mini Kit, Qiagen) and quantified using fluorometric analysis (Qubit dsDNA BR Assay Kit, Invitrogen). Genomes were sequenced with Illumina MiSeq technology to obtain 75-bp reads with an average 80× coverage. SPAdes (version 3.11.1) assembled bacterial genomes de novo from raw reads.

Population structure analysis using core genome MLST scheme

The population structure of the whole species had been determined using a genome-wide approach, using a homemade python script for a cgMLST(https://github.com/bvalot/wgMLST). The genomes of the five PC-*Ck* isolates (Table 1) were compared to those of the 20 U-*Ck* isolates (Table 2) through a cgMLST scheme. A homemade database, with 25 genome data sets, was used to generate the cgMSLT scheme. A total of 4950 nonredundant core genes, present in over 96% (24/25) of the genomes analyzed were included in this scheme. Pairwise allelic profile comparisons built a pairwise distance matrix and a phylogenetic network visualized with FigTree software (version 1.4.4). This phylogenetic reticulation displayed the genetic relation between isolates of the collection and evaluated the distribution of PC-*Ck* isolates throughout the species. Then, a *C. koseri* pan genome (that represents the sum of the core and accessory genomes) have been inferred using the Roary pan genome pipeline, from genomic bacterial sequences annotated beforehand with the command line software tool Prokka using the *C. koseri* Uniprot proteins database.[12,13] Genes specific to PC-*Ck* isolates, defined as those present in all the genomes PC-Ck isolates and absent from the genomes of all U-*Ck*, were sought.

RESULTS

Core genome multi locus sequence typing (cgMLST)

The resulting phylogenetic network from cgMLST-based approach to the collection of 25 isolates used to visualize evolutionary relationships within PC-*Ck* and U-*Ck* suggested that the population of *C. koseri* is nonclonal (Fig. 1A). Nonetheless, cgMLST also identified a cluster A containing three PC-*Ck* isolates (PAR, NAN, and BES) and eight U-*Ck* isolates. The U-*Ck* included in the cluster A were retrieved from various clinical samples (blood culture, urine, vagina, feces) (Table 2). Finally, PC-*Ck* isolates did not share specific genes.

DISCUSSION

Since 2012, the analysis of French hemovigilance reports revealed outbreaks of PC contaminations with *C. koseri*, a bacterial species with limited involvement in human pathology. The analysis of the whole genomes of the contaminating isolates and their comparison with the rest of the bacterial species revealed that they were not epidemic and that they did not share specific genes. This approach is novel in the analysis of blood product contaminants.

Interestingly, the use of platelet storage medium that began in 2005 and has progressively extended nationwide since 2012 would suggest that changes in PC preparation or storage could have provided the risk of contamination of PCs after manufacture, as reported in the United States in 2018. Indeed, using whole genome sequencing, Jones et al.[3] identified environmental sources of contamination with *Acinetobacter calcoaceticus-baumannii* complex and *Staphylococcus saprophyticus* for four cases of septic transfusion reactions reported between May and October 2018. Here, the clustering of the NAN, PAR, and BES isolates in Cluster A (Fig. 1B) could evoke a common environmental origin, but the environment of the preparation or storage of the PC samples had not been sampled. Nonetheless, the phylogenic network built from the genomes of PC-*Ck* and U-*Ck* collected worldwide clearly revealed that PC-*Ck* scattered throughout the species (Fig. 1A). Although the cluster A gathered three PC-Ck isolates, it also included eight U-Ck of various origins and pathogenicity, ruling out the possibility of a PC-*Ck* specific clone (Fig. 1B). The origin of the Cluster A distributed globally raised the possibility of the spread of a *C. koseri* clone that persists in a host population for long periods due to a poor virulence. The further acquisition of antibiotic resistance and virulence genes would explain its involvement in human diseases. Overall, except for the globally distributed Cluster A, the population structure of *C. koseri* is nonclonal, thus ruling out the possibility of the contamination of the PCs from a common source.

The possibility of specific growth features shared by distinct bacterial lineages of *C. koseri* was tested in preliminary experiments. We compared the growth curves of PC-*Ck* and U-*Ck* in PC containing citrate 10 mM brought by the additive solution (AS; PAS III, INTERSOL). In addition, the growth curves of PC-*Ck* were compared in PC stored either in AS and in PC stored in plasma. Our preliminary data showed that PC-*Ck* and U-*Ck* proliferated similarly in PC stored in AS (Fig. S1, available as supporting information in

the online version of this paper) and that PC-*Ck* did not growth systematically better in PC stored in AS than in PC stored in plasma (Fig. S2, available as supporting information in the online version of this paper).

In conclusion, the elevated incidence of PCs contaminated and TTBI involving *C. koseri* in France between 2012 and 2017 was neither due to the dissemination of a clone nor to the dissemination of distinct lineages sharing specific genes. The determinants of the increasing incidence of TTBI due to PC contamination with *C. koseri* remain unknown. However, the recent outbreak of PC contamination with *C. koseri* could be favored by 1) the storage of the whole blood at greater than 18°C up to 24 hours before blood component separation allowing the survival of a small bacterial inoculum, 2) the contact between platelet and white blood cells within the buffy coat after the centrifugation allowing the bacterial transfer between cells, and 3) an increased proportion of healthy carriers of this opportunist pathogen. These hypotheses have to be further tested.

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AVAIBILITY

Sequence Read Archives are available on NCBI (Bioproject: PRJNA529351).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Comparison of the growth in PS-AS of *C. koseri* isolates related (solid lines) or not (dotted lines) with contamination of PC. (A) Pairwise comparison of LIL (PC-*Ck* isolate) versus D7H (U-*Ck* isolate). (B) Pairwise comparison BES (PC-*Ck* isolate) versus D6H (U-*Ck* isolate). Growth curves were drawn from the mean of bacterial counts (n = 2).
Fig. S2. Comparison of the growth of *C. koseri* in PC-AS (dotted lines) and in PC-P (solid line). (A) BES isolate. (B) LIL isolate. (C) MTP isolate. (D) NAN isolate. (E) PAR isolate. Growth curves were deduced from average of duplicate bacterial counts.