What is already known on this topic?
Administration of yellow fever vaccine is contraindicated in children aged <6 months because of increased risk for vaccine-associated encephalitis; the Advisory Committee on Immunization Practices cautions against vaccinating breast-feeding women to avoid the potential risk for transmission of yellow fever vaccine virus to breast-feeding infants.

What is added by this report?
This report describes laboratory-confirmed, breast-feeding–associated transmission of 17DD yellow fever vaccine virus from a recently vaccinated mother; the affected infant developed postvaccinal encephalitis requiring hospitalization.

What are the implications for public health practice?
Health-care personnel should be aware that yellow fever vaccine virus can be transmitted through breast-feeding, and administration of yellow fever vaccine to breast-feeding women should be avoided except in situations where exposure to circulating yellow fever viruses cannot be avoided.

fever vaccines is contraindicated in infants aged <6 months (*4,7,8*).

Yellow fever virus, either wild-type or 17D, has not been reported to have been isolated from or detected in human breast milk. West Nile virus (WNV), another flavivirus, has been detected in milk from WNV-infected, lactating women (*9*), and one case of probable WNV transmission through breast-feeding has been reported (*10*). The actual risk for 17DD virus transmission through breast-feeding cannot be characterized because the number of breast-feeding women who have been vaccinated without negative sequelae in their infants is unknown. Based on the theoretical risk for yellow fever vaccine virus transmission through breast milk, the Advisory Committee on Immunization Practices recommends that yellow fever vaccination of nursing mothers be avoided, except when travel of nursing mothers to high-risk yellow fever–endemic areas cannot be avoided or postponed (*7*). Vaccine recommendations from the World Health Organization do not include considerations for breast-feeding mothers (*8*).

In Brazil, yellow fever vaccination is recommended for all residents of municipalities considered at risk for yellow fever transmission, and for travelers to at-risk areas (*1*). As a result of this investigation, the Brazilian Ministry of Health is revising its recommendations to caution against administration of yellow fever vaccine to breast-feeding women, except in situations where the risk for contracting yellow fever is unavoidable. Further studies on excretion of 17DD virus in breast milk of vaccinated, lactating women would help to define a risk period for viral transmission in cases where vaccination of nursing mothers is necessary.

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別紙様式第 2－1

医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日 2010 年 3 月 3 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称	別紙のとおり	研究報告の公表状況	Infect Genet Evol 9:1240–1247	公表国 フランス		
販売名（企業名）	別紙のとおり					

問題点：フランスの家禽と役場従業員に発生した非定型肺炎に関する調査から、クラミジアの新株の存在が示唆された。

研究報告の概要

フランスの家禽と役場従業員に発生した非定型肺炎に関する調査から、クラミジアの新たな株の存在が示唆された。と役場に家禽を供給した 10 農場における 25 群から得られた検体を用いて PCR 検査を行ったところ、同 25 群の内 14 群にクラミジア関連因子が認められた。同 14 群の内の 1 群の因子は Chlamydophila psittaci と同定されたものの、他の群の因子はこれまでに分類されていないものであった。未分類因子が認められた群の中の異なる 6 群の検体を用いた感染実験の結果、それらの 16S rRNA の遺伝子は非常に近い配列を有し、Chlamydophila 属に属することは明らかであるものの、同属の新たな株である可能性が示唆された。今のところ、これらの因子が人畜共通感染症の感染因子であるかは不明である。

報告企業の意見	今後の対応	使用上の注意記載状況・その他参考事項等
別紙のとおり	今後とも関連情報の収集に努め、本剤の安全性の確保を図っていきたい。	記載なし

MedDRA/J ver.12.1

(10)

別紙

一般的名称	①人血清アルブミン、②人血清アルブミン、③人血清アルブミン*、④人免疫グロブリン、⑤人免疫グロブリン、⑥人免疫グロブリン、⑦乾燥ペプシン処理人免疫グロブリン、⑧乾燥ペプシン処理人免疫グロブリン、⑨乾燥スルホ化人免疫グロブリン、⑩乾燥スルホ化人免疫グロブリン、⑪乾燥スルホ化人免疫グロブリン、⑫乾燥スルホ化人免疫グロブリン、⑬乾燥スルホ化人免疫グロブリン、⑭乾燥スルホ化人免疫グロブリン*、⑮乾燥濃縮人活性化プロテインC、⑯乾燥濃縮人血液凝固第Ⅷ因子、⑰乾燥濃縮人血液凝固第Ⅷ因子、⑱乾燥濃縮人血液凝固第Ⅸ因子、⑲乾燥濃縮人血液凝固第Ⅸ因子、⑳乾燥濃縮人血液凝固第Ⅸ因子、㉑乾燥抗破傷風人免疫グロブリン、㉒乾燥抗破傷風人免疫グロブリン、㉓乾燥濃縮人アンチトロンビンⅢ、㉔ヒスタミン加人免疫グロブリン製剤、㉕フィブリノゲン加第ⅩⅢ因子、㉖乾燥濃縮人アンチトロンビンⅢ、㉗抗 HBs 人免疫グロブリン、㉘抗 HBs 人免疫グロブリン、㉙トロンビン、㉚フィブリノゲン加第ⅩⅢ因子、㉛乾燥濃縮人アンチトロンビンⅢ、㉜乾燥濃縮人アンチトロンビンⅢ、㉝ヒスタミン加人免疫グロブリン製剤、㉞ヒスタミン加人免疫グロブリン製剤、㉟人血清アルブミン*、㊱人血清アルブミン*
販売名（企業名）	①献血アルブミン 20 "化血研"、②献血アルブミン 25 "化血研"、③人血清アルブミン "化血研"*、④ "化血研" ガンマーグロブリン、⑤ガンマーグロブリン筋注 450mg/3mL「化血研」、⑥ガンマーグロブリン筋注 1500mg/10mL「化血研」、⑦献血静注グロブリン "化血研"、⑧献血グロブリン注射用 2500mg「化血研」、⑨献血ベニロン－Ⅰ静注用 500mg、⑩献血ベニロン－Ⅰ静注用 1000mg、⑪献血ベニロン－Ⅰ静注用 2500mg、⑫献血ベニロン－Ⅰ静注用 5000mg、⑬ベニロン*、⑭注射用アナクトC2、500 単位、⑮コンファクトF、⑯コンファクトF注射用 500、⑰コンファクトF注射用 1000、⑱ノバクトM、⑲ノバクトM注射用 250、⑳ノバクトM注射用 500、㉑ノバクトM注射用 1000、㉒テタノセーラ、㉓テタノセーラ筋注用 250 単位、㉔ヘパトセーラ、㉕ヘパトセーラ筋注 200 単位/mL、㉖トロンビン "化血研"、㉗ボルヒール、㉘ボルヒール組織接着用、㉙アンスロビンP、㉚アンスロビンP500 注射用、㉛ヒスタグロビン皮下注用、㉜アルブミン 20%化血研*、㉝アルブミン 5%化血研*、㉞静注グロブリン*、㉟アンスロビンP1500 注射用
報告企業の意見	クラミジア（Chlamydia）は 300nm 程度の大きさで、細胞内でのみ増殖する偏性細胞内寄生微生物であり、DNA と RNA を有し、2 分裂で増殖する。今回の報告は家禽と殺場従業員に発生した非定型肺炎に関する調査を機に、クラミジアの Chlamydophila 属における新株の可能性が示唆されたものであるが、それらが人畜共通感染症の感染因子であるかは不明である。 弊所で製造している全ての血漿分画製剤の製造工程には、約 0.2μm の無菌ろ過工程および、クラミジアよりも小さいウイルスの除去を目的としたウイルス除去膜ろ過工程が導入されているので、仮に製造原料にクラミジアが混入していたとしても、これらの工程により除去されるものと考えられる。更に、これまでに上記製剤によるクラミジア感染の報告例は無い。 以上の点から、上記製剤はクラミジア感染に対する安全性を確保していると考える。

*現在製造を行っていない

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Isolation of a new chlamydial agent from infected domestic poultry coincided with cases of atypical pneumonia among slaughterhouse workers in France

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ABSTRACT

Three cases of atypical pneumonia in individuals working at a poultry slaughterhouse prompted an epidemiological survey in 10 poultry farms that had supplied birds. Using a *Chlamydiaceae*-specific real-time PCR assay, chlamydial agents were detected in 14 of 25 investigated flocks. Rather unexpectedly, *Chlamydophila psittaci* was identified only in one of the positive flocks, whereas ArrayTube DNA microarray testing indicated the presence of a new, so far unclassified member of the genus *Chlamydophila*.

For further characterization of the agent involved, positive cloacal swabs were used to inoculate embryonated chicken eggs and isolates were obtained from 6 different flocks. Sequencing of 16S rRNA genes revealed nearly identical sequences of all samples. Alignment with representative sequences of *Chlamydiaceae* showed the separate position of the present strains outside the currently recognized species of *Chlamydophila*, but clearly within this genus. In contrast, partial *ompA* gene sequences displayed considerable diversity among the isolates, which had already been observed in restriction enzyme analysis of *ompA* PCR products. These data suggest that each farm had been infected with a different strain of this new chlamydial agent, the zoonotic potential and the exact taxonomic status of which have yet to be defined.

1. Introduction

Chlamydial infections leading to outbreaks of avian chlamydiosis in domestic, companion and wild birds are regularly reported from all parts of the world. As their general importance is based on two aspects, i.e. economic losses to the bird owners and potential zoonotic transmission to humans, control measures are obligatory in a number of European countries, where specific state legislation is in force. The most prominent chlamydial agent in *Aves* is *Chlamydophila* (*C.*) *psittaci*, which was shown to occur in as many as 465 bird species (Kaleta and Taday, 2003). Following the recent revision of chlamydial taxonomy (Everett et al., 1999), this obligate intracellular bacterium now predominantly comprises avian serovars. The family *Chlamydiaceae* with its two genera *Chlamydia* and *Chlamydophila* currently combines a total of nine species, i.e. *Chlamydia trachomatis*, *Chlamydia suis* and *Chlamydia muridarum*, as well as *C. pneumoniae*, *C. abortus*, *C. caviae*, *C. felis*, *C. pecorum*, and *C. psittaci*, respectively.

The importance of *C. psittaci* as the causative agent of psittacosis or avian chlamydiosis in psittacine birds and domestic fowl has been known for decades. A number of recent reports have confirmed that its zoonotic potential remains significant in the face of regularly occurring outbreaks of disease in domestic fowl (Vanrompay et al., 1995; Gaede et al., 2008; Laroucau et al., 2009). In addition, infections can take a subclinical and/or chronic course (Harkinezhad et al., 2009). However, occasional detections of *C. abortus* (Herrmann et al., 2000; Pantchev et al., 2008) and so far non-classified chlamydial agents (Gaede et al., 2008), as well as genetic evidence on intermediate strains between *C. psittaci* and *C. abortus* (Van Loock et al., 2003) suggest that the spectrum of *Chlamydiaceae* spp. encountered in birds is not confined to a single species.

In this context, it should be noted that laboratory diagnosis of infections involving chlamydiae has undergone a remarkable methodological change in the past two decades (Sachse et al., 2009). While only a few specialized laboratories are still conducting routine isolation of chlamydiae using cell culture or embryonated eggs, DNA-based detection methods have become widely accepted. This implies that specific PCR tests for individual chlamydial species and/or pan-*Chlamydiaceae* assays are conducted, which are capable of detecting even small amounts of a known agent within a working day. However, new and hitherto

Abbreviations: MOMP, major outer membrane protein; RFLP, restriction fragment length polymorphism; rtPCR, real-time PCR.

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non-classified taxa will often be overlooked by this approach, so that the inclusion of a highly parallel screening assay, e.g. a DNA microarray test, is advisable.

Following the occurrence of symptoms of atypical pneumonia in a group of poultry slaughterhouse workers in France early in 2008, a diagnostic investigation in the slaughterhouse and the flocks of the suppliers was undertaken. Initial tests suggested involvement of chlamydiae, so that diagnostic examinations focused on *Chlamydiaceae* spp. In the present paper, we report the results of this study, which led to the identification of so far non-classified avian chlamydial agents.

2. Materials and methods

2.1. History of human infections

In the period from April 25 to May 30 in 2008, three individuals presented to their physicians with atypical pneumonia. Case 1, a 49-year-old woman, had fever and flu-like symptoms and was administered Naxy. Case 2 was a 25-year-old woman suffering from thoracic pain and fever. She had to be hospitalized and was treated with Clamoxyl and Rulide. Case 3, a man aged 62, had fever and complained about weariness. He was administered Zeclar. No diagnostic data from microbiology and serology are available.

2.2. Animal samples

Cloacal swabs were collected from poultry flocks received for slaughter and from birds belonging to the slaughterhouse owner. In each sampled flock, 10 animals were examined. One panel of swabs was stored in 1 ml of conservation buffer SPG (Spencer and Johnson, 1983) at −80 °C until inoculated into chicken eggs, whereas the other panel was stored dry at −80 °C until subjected to DNA extraction. Data on age, breed and flock of the examined birds are contained in Table 1.

2.3. DNA extraction

Dry cloacal swabs were subjected to DNA extraction using the QIAamp DNA Mini Kit (QIAGEN, Courtaboeuf, France) following the buccal swab protocol. For DNA extraction from aliquots of chlamydial cell culture and vitellus membranes, the tissue protocol of the same kit was used. Finally, DNA was eluted with 150 μl of AE buffer and stored at −20 °C until examination.

2.4. Direct detection of chlamydiae by real-time PCR

A *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene (23S-rtPCR) was used in this study (Ehricht et al., 2006). The protocol includes primers Ch23S-F (5′-CTGAAACCAGTAGCTTA-TAAGCGGT-3′), Ch23S-R (5′-ACCTCGCCGTTTAACTTAACTCC-3′), and probe Ch23S-p (FAM-5′-CTCATCATGCAAAAGGCACGCCG-3′-TAMRA). Each reaction mix contained 2 μl sample DNA template, 10 μl of Universal Mastermix 2× (Applied Biosystems, Courtaboeuf, France), 0.5 μl of each primer (25 μM), 2 μl of the probe (1 μM), and 5 μl deionized water. The temperature–time profile was 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 60 s.

The *ompA*-based real-time PCR assay specific for *C. psittaci* was conducted as recently described (Pantchev et al., 2008).

2.5. Culture on embryonated chicken eggs

For culture, suspensions of cloacal swabs stored in conservation buffer at −80 °C were thawed, transferred into sterile Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. Supernatant was transferred into a new sterile tube. Antibiotic solution containing 0.1 mg of vancomycin, 0.1 mg of streptomycin, 0.1 mg of kanamycin and 100 U of nystatin was added to the supernatant and the pellet suspensions, which were then incubated at 37 °C for 2 h before inoculation.

Yolk sacs of 7-day-old embryonated eggs were inoculated with 0.2 ml per egg, and five eggs per sample. For each set of inoculation, three eggs were inoculated with *C. psittaci* strain Loth as positive control, and three other eggs were kept separately as non-infected controls. Eggs were incubated at 38 °C and observed daily.

Vitellus membranes were collected and analyzed using a MIF test, i.e. a direct *immunofluorescence assay* (Chlamydia direct IF, BioMérieux, Marcy l'Etoile, France). One of the two monoclonal antibodies contained in this kit allowed the identification of *Chlamydiaceae* spp.

Table 1
Characteristics of investigated poultry flocks and results of diagnostic testing.

Flock ID	Breeder no.	Flock effective	Breed	Age at time of sampling (weeks)	23S-rtPCR *Chlamydiaceae*		*ompA*-rtPCR *C. psittaci*	Cell culture isolation
					No. of +ve samples	Mean Ct	No. of +ve samples	
Slaughterhouse								
08-1274/2	Slaughterhouse	500	Chicken	4	1/10 (10%)	38.6	0/1	
08-1274/1	breeder	500	Chicken	8	0/10	–	–	
08-1274/5		500	Chicken	12	0/10	–	–	
08-1274/4		500	Chicken	16	4/10 (40%)	33.0	0/4	
08-1274/3		500	Chicken	20	10/10 (100%)	28.8	0/10	+
08-1274/6		100	Guinea fowl	7	0/10	–	–	
08-1274/8		120	Guinea fowl	13	0/10	–	–	
08-1274/7		120	Guinea fowl	16	0/10	–	–	
Customers								
08-1274/9	Breeder 1	320	Chicken	20	3/10 (30%)	34.6	0/3	
08-1274/10		–	Guinea fowl	20	0/10	–	–	
08-1274/17		–	Duck	–	1/5 (20%)	35.1	1/1	+
08-1274/11	Breeder 2	600	Chicken	15	0/10	–	–	
08-1274/12		–	Guinea fowl	15	1/8 (12.5%)	40.1	0/1	
08-1274/13	Breeder 3[a]	600	Chicken	–	9/9 (100%)	26.3	0/9	+
08-1274/23		600	Chicken	12	10/10 (100%)	27.2	0/10	+
08-1274/24		600	Chicken	4	3/10 (30%)	39.0	0/3	
08-1274/14	Breeder 4	–	Chicken	–	0/10	–	–	
08-1274/15		–	Guinea fowl	–	0/8	–	–	
08-1274/16	Breeder 5[a]	600	Chicken	8	1/10 (10%)	38.8	0/1	
08-1274/19		600	Chicken	18	10/10 (100%)	27.0	0/10	+
08-1274/18	Breeder 6[a]	600	Chicken	18	0/10	–	–	
08-1274/20	Breeder 7[a]	600	Chicken	12	1/10 (10%)	38.9	0/1	
08-1274/21		600	Chicken	17	10/10 (100%)	26.6	0/10	+
08-1274/22	Breeder 8[a]	600	Chicken	16	9/10 (90%)	31.8	0/9	+
08-1274/25	Reproducer	407	Chicken	43	0/10	–	–	

[a] Breeders linked to the same supplier of 1-day-old chickens, i.e. 08-1274/25.

Table 2
Primers used for sequencing.

Targeted sequence	Primer name	Sequence[a] (5′-3′)	PCR product size	References
16S rRNA	16S1	CGGATCCTGAGAATTTGATC	1200 bp	Pudjatmoko et al. (1997) Thomas et al. (2006)
	rp2	CTACCTTGTTACGACTTCAT		
ompA	191CHOMP	GCIYTTTCGGGARTGYGGITGYGCIAC	600 bp	Sachse and Hotzel (2003)
	CHOMP371	TTAGAAICKGAATTGIGCRTTIAYGTGIGCIGC		

[a] Degenerate nucleotides: K = G, T; M = A, C; W = A; T; Y = C, T; I = Inosine.

2.6. Cell culture

Chlamydial isolates were grown in Buffalo Green Monkey (BGM) kidney cells as previously described (Sachse et al., 2003). Three days after inoculation, coverslips were fixed with methanol, and the monolayer was stained using the IMAGEN™ Chlamydia kit (DAKO Ltd., Cambridgeshire, UK). For confocal laser scanning microscopy using a TCS-SP2 confocal microscope (Leica, Bensheim, Germany), the coverslips were stained with Evans Blue, FITC-labeled anti-Chlamydia antibody (Oxoid, Cambridgeshire, UK) and DAPI (Sigma, Taufkirchen, Germany).

2.7. DNA-based characterization

2.7.1. DNA microarray testing

The ArrayTube™ (AT) assay was used as described (Borel et al., 2008) to check samples for the presence of DNA from any of the currently accepted nine species of *Chlamydia* and *Chlamydophila*, as well as *Waddlia chondrophila* and *Simkania negevensis*. Briefly, DNA template was amplified and biotin labeled in 40 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/30 s, using primers U23F-19 (5′-ATTGAMAGGCGAWGAAGGA-3′) and 23R-22 (5′-biotin-GCYTAC-TAAGATGTTTCAGTTC-3′). Hybridization was conducted in the AT vessel at 58 °C for 1 h. Subsequently, the array was washed, and hybridization patterns were visualized using streptavidin-conjugated peroxidase-catalyzed precipitation and processed using the ATR-01 reader (Clondiag Chip Technologies, Jena, Germany) and the Iconoclust 2.3 program (Clondiag).

2.7.2. Multiple locus VNTR analysis

Samples were examined using the recently published MLVA procedure (Laroucau et al., 2008a).

2.7.3. Amplifications for DNA sequencing

Primers used for the partial amplification of *ompA* and 16S rRNA genes are listed in Table 2. PCR was performed in a total volume of 50 μl containing 4 μl of DNA template, 5 μl of 10× PCR reaction buffer, 2 U of Hot start *Taq* DNA polymerase (Qiagen), 400 μM of each deoxynucleotide triphosphate, and 0.5 μM of each flanking primer. The temperature–time profile for amplification of the 16S rRNA gene was 95 °C for 10 min, 4 cycles of 95 °C for 1 min, 40 °C for 1 min, 72 °C for 2 min and 36 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min. The final extension step was at 72 °C for 10 min.

2.7.4. Genotyping using restriction fragment length polymorphism (RFLP) of ompA amplicons

Aliquots of 10 μl of PCR product (primers 191CHOMP/CHOMP371, from Sachse and Hotzel, 2003) were subjected to overnight restriction enzyme digestion using 1 U of *AluI* (Euromedex, Souffelweyersheim, France) in a total volume of 15 μl at 37 °C. The digestion products were then loaded onto a 4% gel (50% Nusieve–50% Metaphore) containing ethidium bromide and analyzed using a UV transilluminator.

2.7.5. DNA sequencing

PCR-amplified segments of *ompA* and 16S rRNA genes were purified using the QIAquick PCR Purification Kit (Qiagen). DNA sequencing was done at MWG Biotech France (Roissy, France). Nucleotide sequences have been deposited under GenBank no. GQ398026 to GQ398031 for 16S rRNA sequences and under GenBank no. GQ398032 to GQ398038 for *ompA* sequences.

2.7.6. Sequence analysis

All 16S rRNA and *ompA* gene sequences determined in this study were combined in an alignment with previously published sequences that were relevant for phylogenetic and epidemiological considerations. Accession numbers of these sequences are listed in Table 3. Sequence data were analyzed using the Bionumerics software package version 4.6 (Applied-Maths, Saint-Martens-Latem, Belgium) as a character dataset. Cluster analysis was conducted using the categorical parameter and the UPGMA coefficient.

3. Results

3.1. Examination of samples from infected flocks

In preliminary examinations following diagnosis of atypical pneumonia in humans, two working-surface area samples from the slaughterhouse where those workers were employed, 20 swabs collected from chickens and guinea fowl belonging to the flock of the slaughterhouse owner and two fecal samples from the same flock were tested by real-time PCR. The results given in Table 4 revealed the presence of *Chlamydiaceae* in the birds as well as in the slaughterhouse itself. Quite surprisingly, none of the samples was positive for *C. psittaci*.

Subsequently, samples were collected from 16 poultry flocks, all of which were regular suppliers to this slaughterhouse. The prevailing bird species were chickens of the "Barbezieux" and "Coucou" breeds, but guinea fowl (three flocks) and ducks (one flock) were also included. In addition, samples were taken from 8 flocks belonging to the slaughterhouse owner and the flock of the egg supplier of five breeders raising "Barbezieux" chickens (Breeders no. 3, 5, 6, 7 and 8). Essential data and testing results are given in Table 1. In real-time PCR examinations, *Chlamydiaceae* were detected in 14 flocks. Comparison of Ct values indicated different

Table 3
BLAST analysis of partial *ompA* sequences (about 480 nt) of samples from flocks 08-1274/3, 08-1274/9, 08-1274/13, 08-1274/19, 08-1274/21 and 08-1274/22.

Origin of strain	*ompA* partial fragment size (nt)	GenBank acc. no. of partial *ompA* sequence	Highest similarity to (GenBank acc. no.)	Total BLAST score	Query coverage	E value	Max identity
08-1274/3	480 bp	GQ398033	*Chlamydophila* sp. 6617-T5 (EU019096)	479	60%	6.00E−132	96%
			Chlamydophila sp. 6620-T4 (EU019095)	479	60%	6.00E−132	96%
			Chlamydophila sp. 6688-T2 (EU019094)	440	62%	3.00E−120	93%
08-1274/9 (swab)	489 bp	GQ398034	*Chlamydophila* sp. 6617-T5 (EU019096)	695	77%	0	99%
			Chlamydophila sp. 6620-T4 (EU019095)	686	76%	0	99%
			Chlamydophila sp. 6688-T2 (EU019094)	455	60%	1.00E−124	94%
			Chlamydophila sp. PEENT (U82955)	324	59%	3.00E−85	86%
08-1274/13 & 08-1274/23	480 bp	GQ398035, GQ398032	*Chlamydophila* sp. 6617-T5 (EU019096)	484	60%	1.00E−133	96%
			Chlamydophila sp. 6620-T4 (EU019095)	484	60%	1.00E−133	96%
			Chlamydophila sp. 6688-T2 (EU019094)	457	62%	3.00E−125	94%
08-1274/19	486 bp	GQ398036	*Chlamydophila felis* MOMP gene for major outer membrane protein (X61096)	182	30%	2.00E−42	89%
08-1274/21	489 bp	GQ398037	*Chlamydophila* sp. 6617-T5 (EU019096)	363	76%	6.00E−97	84%
			Chlamydophila sp. 6620-T4 (EU019095)	359	76%	8.00E−96	84%
			Chlamydophila sp. PEENT (U82955)	353	61%	4.00E−94	88%
			Chlamydophila sp. 6688-T2 (EU019094)	348	75%	2.00E−92	84%
08-1274/22	483 bp	GQ398038	*Chlamydophila* sp. 6688-T2 (EU019094)	320	74%	4.00E−84	82%
			Chlamydophila felis MOMP gene for major outer membrane protein (X61096)	204	72%	4.00E−49	77%

Table 4
Summary of preliminary diagnostic testing.

1st investigation	No. of analyzed samples	23S-rtPCR *Chlamydiaceae* No. of positive samples	*ompA*-rtPCR *C. psittaci* no. of positive samples
In the slaughterhouse	2	1/2	0/1
In the personal flock of the owner (chickens and guineafowl)			
Cloacal swabs	20	5/20	0/5
Fecal samples	2	1/2	0/1

levels of chlamydial excretion, with birds from flocks no. 3, 13, 19, 21, 22, and 23 being identified as high excretors and with almost 100% of animals testing positive. Notably, flock 08-1274/25, which was linked with flocks 08-1274/13, 08-1274/16, 08-1274/18, 08-1274/19, 08-1274/20, 08-1274/21, 08-1274/22, 08-1274/23 and 08-1274/24 for being their exclusive supplier of 1-day-old chicks, proved negative when tested.

Again, *C. psittaci* was not the predominant chlamydial agent, since only a single sample (from flock no. 08-1274/17, the only investigated duck flock) was positive in *C. psittaci*-specific real-time PCR and, subsequently, also in MLVA. The *C. psittaci*-specific MLVA genotype of the duck sample was 2:3:2:0:6:3:0:4 using primers ChlaPsi_280, ChlaPsi_480, ChlaPsi_605, ChlaPsi_810, ChlaPsi_222, ChlaPsi_281, ChlaPsi_929 and ChlaPsi_1778, respectively (data not shown).

3.2. Direct genotyping of clinical samples

While PCR using the classical *ompA* primers CTU/CTL (Denamur et al., 1991) failed to produce amplicons from positive non-*C. psittaci* samples (data not shown), the use of degenerate primers 191CHOMP/CHOMP371 enabled further characterization by RFLP. DNA extracts from real-time PCR-positive (Ct < 34) cloacal swabs were subjected to PCR and digested with *AluI*. As shown in Fig. 1, all restriction patterns within a flock were identical, but clearly differed among flocks 08-1274/3, 08-1274/9, 08-1274/13, 08-1274/21, 08-1274/22, and 08-1274/19. As the only exception, patterns from flocks 08-1274/3 and 08-1274/4 were identical. These 2 flocks belonged to the same breeder (i.e. the owner of the slaughterhouse, see Table 1). The same was observed for flocks 08-1274/13 and 08-1274/23, which also belonged to the same breeder (Breeder no. 3).

3.3. DNA-based characterization of isolated strains

In cell culture trials, duplicates of the PCR-positive dry swabs, which had been stored in SPG medium, were inoculated into embryonated chicken eggs. Isolates were successfully cultured from flocks 08-1274/3, 08-1274/13, 08-1274/19, 08-1274/21, 08-1274/22 and 08-1274/23 (Table 1). The same strains also grew well

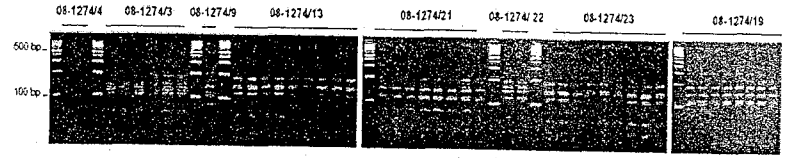

Fig. 1. Genotyping of cloacal swab samples by RFLP. *AluI* enzyme restriction profiles of partial *ompA* PCR products from clinical samples of 8 flocks. The following samples from the respective flocks were examined: 08-1274/4 (2 birds), 08-1274/3 (6 birds), 08-1274/9 (1 bird), 08-1274/13 (9 birds), 08-1274/21 (8 birds), 08-1274/22 (2 birds), 08-1274/23 (9 birds) and 08-1274/19 (8 birds). DNA size marker (GeneRuler™ 100 bp, Euromedex, France) was loaded between each flock. Fragment sizes (in bp) are given on the left-hand margin.

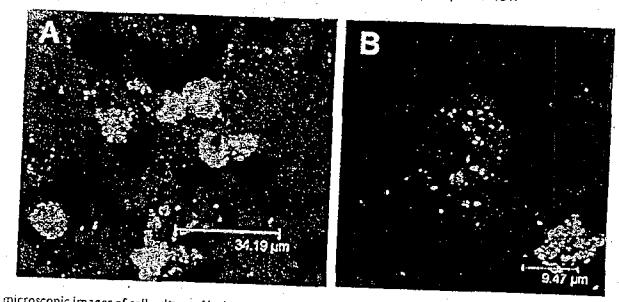

Fig. 2. Confocal laser scanning microscopic images of cell culture of isolate 08-1274/3. The chlamydial agent was grown in BGM cells, fixed with methanol on coverslips, and the monolayer was stained using Evans Blue for BGM cells (red color), FITC-labeled anti-Chlamydia antibody for chlamydial bodies (green, A) and DAPI for BGM cell nuclei (blue, A). Yellow: co-localization of chlamydial inclusions and cellular Evans Blue (A). FITC-labeled anti-Chlamydia antibody for chlamydial bodies (B). Scale bars are given on each image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in cell culture of BGM cells, where intracellular inclusions reminiscent of other chlamydial agents were observed (Fig. 2). DNA extracts from these cultures were examined by DNA microarray analysis using the AT test. Fig. 3 shows a typical hybridization pattern, where only the genus-specific probes generated specific positive signals, whereas species-specific signals were absent. These results indicated that DNA of any of the 9 established species of *Chlamydiaceae* was not present in the samples.

To establish the identity of the strains encountered in the poultry flocks, partial sequencing of the *ompA* gene was conducted for 6 isolates (from flocks 08-1274/3, 08-1274/13, 08-1274/19, 08-1274/21 08-1274/22 and 08-1274/23) and one swab sample from flock 08-1274/9. BLAST analysis shown in Table 3 revealed that *ompA* sequences of samples from flocks 08-1274/3, 08-1274/9 and 08-1274/13 exhibited the highest degree of similarity to a group of sequences from an outbreak of psittacosis in Germany (EU019094–EU019096), which had been tentatively classified as *Chlamydophila* spp. because they could not be assigned to any of the currently defined species (Gaede et al., 2008). Furthermore, *ompA* sequences of isolates from flocks 08-1274/9 and 08-1274/21 were found to have moderate similarity to another non-classified strain of *Chlamydophila* spp. (U82955) isolated from a peacock. The isolates from flocks 08-1274/19 and 08-1274/22 displayed moderate similarity to the *C. felis* ompA sequence and to one of the group of sequences from an outbreak of psittacosis in Germany, respectively. Sequences from isolates 08-1274/13 and 08-1274/23 (same breeder) were identical.

To explore the genetic relatedness of the strains from the present study, all sequences mentioned in Table 3 were aligned

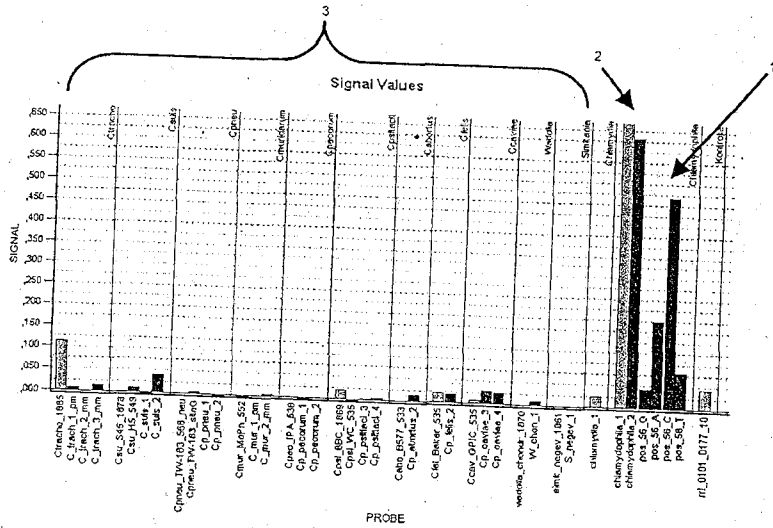

Fig. 3. Hybridization pattern obtained from examination of isolate 08-1274/3 using the ArrayTube DNA microarray assay. Barplot of hybridization signals: 1 consensus probe (family *Chlamydiaceae*), 2 genus-specific probes (*Chlamydophila*), 3 probes specific for the currently defined nine species of *Chlamydiaceae*.

with *ompA* sequences of strains representing the established species of *Chlamydia* and *Chlamydophila*. The dendrogram shown in Supplement 1 indicates that the strains described here form a separate cluster situated at the margin of the genus *Chlamydophila*.

As the *ompA* gene generally is distinguished by high intra-species diversity among chlamydiae, analysis of the more conserved 16S rRNA gene sequences was conducted to obtain alternative information on the identity and characterize the

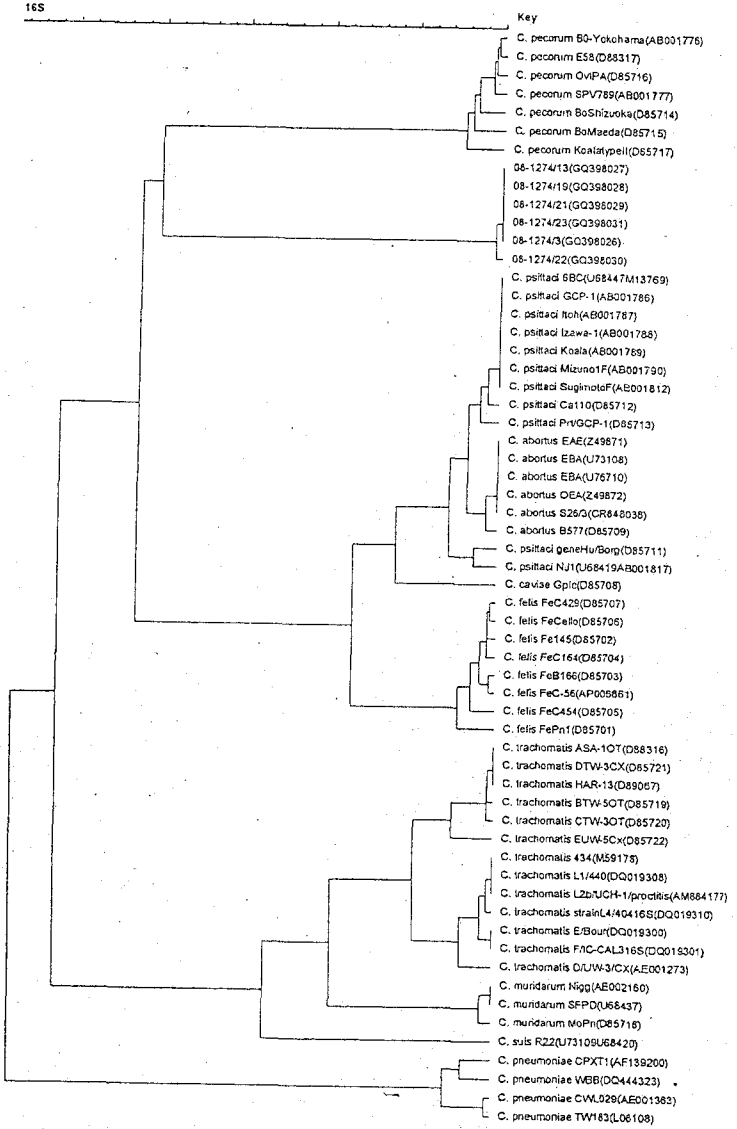

Fig. 4. Dendrogram for *Chlamydiaceae* based on a partial sequence analysis 16S rRNA genes. The tree was constructed by the neighbour-joining method from phylogenetic distances calculated by UPGMA method. Horizontal distances correspond to genetic distances, vertical distances are arbitrary.

taxonomic position of the present isolates. Segments of 1436 nt representing nearly the entire gene were sequenced from samples from the five strains and one clinical sample mentioned in Table 4. All sequences obtained were identical except for a single point mutation in 08-1274/22. Alignment with representative sequences of *Chlamydiaceae* confirmed the separate position of the present strains outside the existing species of *Chlamydophila*, but clearly within the genus. The corresponding dendrogram is shown in Fig. 4. Sequence similarity values given in Supplement 2 also clearly show the distinct genetic position of the present strains.

4. Discussion

So far, very few studies on avian chlamydiosis in chickens have been conducted in France and elsewhere. Veterinary investigations are usually undertaken when transmission to humans is suspected. To obtain more information, an epidemiological study on psittacosis involving 15 French administrative units is currently underway (http://www.invs.sante.fr/surveillance/psittacose/default.htm), whose aim is the determination of the incidence of hospitalized human cases, as well as the frequency of grouped cases, and risk assessment for exposed individuals. Additionally, the analysis of strains isolated from humans and animals and the description of breeding characteristics and working conditions should improve the knowledge on risk factors for animal-to-human transmission. Up to now, most of the recently confirmed cases of *C. psittaci* infection have been associated with ducks or exotic birds (Laroucau et al., 2008b, and unpublished data).

The present survey was prompted by the occurrence of hitherto unexplained atypical pneumonia in three French slaughterhouse workers in 2008. This poultry slaughterhouse had originally not been included in the national study mentioned above, but a veterinary survey was started as symptoms of the workers were reminiscent of psittacosis, without any microbiological confirmation.

Although no clinical signs were seen in the birds, diagnostic testing revealed the presence of *Chlamydiaceae* in most of the poultry flocks investigated, and some of the flocks were identified as high excretors. The levels of excretion were similar to those previously observed in *C. psittaci*-infected duck flocks, some of them associated with human infections (Laroucau et al., 2009). Rather unexpectedly, only one out of 73 *Chlamydiaceae*-positive samples of the present panel proved positive in *C. psittaci*-specific real-time PCR. When genotyping of the chlamydial strains involved was attempted, *C. psittaci*-specific VNTR primers failed to generate patterns characteristic for this species (except for the positive sample). Subsequently, the AT test revealed aberrant hybridization patterns, i.e. signals of the genus-specific probes for *Chlamydophila*, but the absence of grouped species-specific signals (Fig. 3). This combined evidence suggested that we were probably dealing with a novel chlamydial agent.

Analysis by RFLP of *ompA* gene segments directly amplified from the most high-titer real-time PCR-positive samples indicated the presence of a single strain within each investigated flock, but also revealed that the strains were different from each other. Thus, 6 isolates were obtained from 6 different flocks raising "barbezieux" or "cou nu" breeds. Two of them, which were isolated at the same farm, proved identical based on their partial *ompA* gene sequences (flocks 08-1274/13 and 08-1274/23). Partial sequencing of the 16S rRNA and *ompA* genes revealed that, while 16S rRNA gene sequences were highly similar among the isolates, the *ompA* sequences were distinguished by high inter-strain heterogeneity. This confirms observations by Everett et al. (1999), who pointed out that rRNA genes were subjected to evolutionary pressure to a far lesser extent than genes encoding outer membrane proteins, such as *ompA*. The same authors recommended that, in order to be classified as a member of *Chlamydiaceae*, a taxon should have less than 10% 16S rRNA gene diversity to any other member of the family. This condition is fulfilled for the isolates described in this study. Furthermore, comparison of the 16S rRNA sequences with those of the established species of *Chlamydiaceae* showed that the present avian strains formed a separate cluster within the genus *Chlamydophila* (Fig. 4). While the evidence gathered so far indicates that the 6 isolates belong to a new species, the authors are aware that more DNA sequence data, as well as morphological and other phenotypic data, are required to justify the definition of a new taxon. To address the epidemiological importance, we will further pursue the question whether these new microorganisms are occurring in other regions and countries. In any case, the present idea of taxonomic classification is still preliminary.

The present investigations were conducted in a limited geographical area, which involved, among others, a small poultry production unit dedicated to the barbezieux chicken breed. This chain involved a unique breeder (parental) flock which supplied 5 other breeders (Breeders no. 3, 5, 6, 7 and 8) dealing with fattening. The birds were all slaughtered in the slaughterhouse concerned. The first surprise was that one of the five breeders (Breeder no. 6) was not affected by these new *Chlamydophila* bacteria (Table 1). Six months later, follow-up sampling was done in another flock of this breeder, and, again, no *Chlamydiaceae* were detected in 10 animals randomly selected and examined 5 times in 16 weeks (at 3, 6, 8, 12 and 16 weeks of age, data not shown). Another surprise was the finding that the four farms having infected flocks harbored different strains, as shown by partial *ompA* gene sequencing, and that the strains were apparently associated to an individual farm. Indeed, sampling another flock of Breeder no. 5 confirmed the presence of one unique strain per farm based on partial *ompA* gene sequences (data not shown). In these circumstances, the source of infection remains unclear, but vertical transmission can be ruled out. It should be noted that the new *Chlamydophila* strains have also been detected in the "cou nu" breed of chicken (flocks 08-1274/3 and 08-1274/9). Interestingly, no *Chlamydiaceae* were found in guinea fowl ($n = 60$), although these birds were mixed with proven positive chickens (flocks 08-1274/2, 08-1274/3 and 08-1274/4).

Notably, no clinical signs were observed in animals of the respective flocks. Autopsy conducted on five birds in flocks 08-1274/21 and 08-1274/23, did not reveal any macroscopic lesions, despite the fact that PCR examination of spleen, liver, lung, and intestinal tissue samples demonstrated an intensive and dominant colonization of intestinal tissue with the new *Chlamydiaceae* spp. in comparison to the other organs that tested weakly positive (data not shown). This is in agreement with the observation of Gaede et al. (2008) that genetically related non-classified *Chlamydophila* spp. were found in symptomless chickens during an outbreak of clinical psittacosis.

The etiological importance of these new chlamydial isolates for human pneumonia has yet to be defined. So far, there has been no hard evidence of the strains being responsible for the three reported human cases of atypical pneumonia. Although clinical signs were reminiscent of psittacosis, i.e. high fever and pulmonary invasion, they cannot be regarded as specific. The three affected individuals were successfully treated with macrolide antibiotics (see Section 2). A possible approach to the assessment of the pathogenicity of the present strains could include experimental challenge trials in mice based on the protocol of Rodolakis et al. (1989).

Finally, it should be emphasized that detection of the presumed new members of *Chlamydiaceae* became possible because of the use of advanced yet complementary DNA-based diagnostic methods, i.e. real-time PCR in conjunction with the AT test. This combination, which was already suggested as a reference standard

(Sachse et al., 2009), can be further recommended for the laboratory diagnosis of animal and human chlamydial infections.

Conflict of interest statement

None of the authors (K. Laroucau, F. Vorimore, R. Aaziz, A. Berndt, E. Schubert, K. Sachse) has a financial or personal relationship with other people or organisations that could inappropriately influence or bias this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2009.08.005.

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No. 27

医薬品 研究報告 調査報告書

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一般的名称	新鮮凍結人血漿					
販売名(企業名)	新鮮凍結血漿「日赤」(日本赤十字社) 新鮮凍結血漿-LR「日赤」(日本赤十字社) 新鮮凍結血漿-LR「日赤」成分採血(日本赤十字社)		研究報告の公表状況	Satake M, Mitani T, Oikawa S, Nagumo H, Sugiura S, Tateyama H, Awakihara S, Mitsutomi Y, Muraoka M, Tadokoro K. Transfusion. 2009 Oct;49(10):2152-7.	公表国 日本	

研究報告の概要	○日本における初流血除去導入前後の血小板濃厚液の細菌汚染頻度 背景：血小板濃厚液(PC)の細菌汚染は、輸血医学における重大な感染リスクとなっている。細菌汚染を低減させる新しい戦略実施の必要性を評価するために、PCの正確な汚染頻度を明らかにすることが必要である。 試験デザインおよび方法：日本赤十字社が供給するPCの細菌汚染頻度を、初流血除去実施前後の有効期限切れPCを用いて調べた。偽陰性結果が可能な限り最少となる培養法をデザインした：保存から4日以上後に血小板検体をサンプリングし、好気性ボトルと嫌気性ボトルの双方に10mLの量を接種した。 結果：初流血除去実施前では、培養したPC21,786検体のうち36(0.17%)に細菌汚染があったことが確認された。実施後の汚染PC数は、21,783中11(0.05%)に減少し(減少率71%)、臨床的に重大な細菌の汚染の件数は、*Propionibacterium acnes*陽性PCを除き4件(0.018%)であった。献血者の血液に由来すると推定される細菌の汚染頻度は減少しなかった。 結論：初流血除去は細菌汚染頻度に確かな影響を及ぼす。西欧諸国と同等の細菌汚染頻度であり、培養スクリーニング検査が実施されていないにもかかわらず、日本でPC輸血後の敗血症反応発現率が低いことは、PCの保存期間が短い(72時間)ことが重要であると示唆される。
報告企業の意見	今後の対応
日本における献血血液の初流血除去導入についての評価報告である。血小板濃厚液の細菌汚染頻度は、初流血除去導入前後で0.17%から0.05%に減少し、臨床的に重大な細菌の汚染の件数は4件(0.018%)であった。培養スクリーニング検査が実施されていないにもかかわらず、輸血後の敗血症発現率が低いことは、PCの保存期間が短いことが重要であると示唆された日本赤十字社の安全対策に関する報告である。	日本赤十字社では、輸血による細菌感染予防対策として、すべての輸血用血液製剤を対象に保存前白血球除去及び初流血除去を導入している。さらに、輸血情報レーブレット等により細菌感染やウイルス感染について医療機関へ情報提供し注意を喚起しているほか、細菌感染が疑われる場合の対応を周知している。細菌やウイルスの検出や不活化する方策について検討している。

使用上の注意記載状況・その他参考事項等	研究報告の概要に関連する情報
新鮮凍結血漿「日赤」 新鮮凍結血漿-LR「日赤」 新鮮凍結血漿-LR「日赤」成分採血	血液を介するウイルス、細菌、原虫等の感染 vCJD等の伝播のリスク

Frequency of bacterial contamination of platelet concentrates before and after introduction of diversion method in Japan

Masahiro Satake, Takako Mitani, Shinji Oikawa, Hideto Nagumo, Sayoko Sugiura, Hidemi Tateyama, Syuji Awakihara, Yoshiro Mitsutomi, Masato Muraoka, and Kenji Tadokoro

BACKGROUND: Bacterial contamination of platelet concentrates (PCs) is the major infectious risk in transfusion medicine. To evaluate the necessity of implementing novel strategies for the reduction of bacterial contamination, it is necessary to establish a precise contamination frequency in PCs.

STUDY DESIGN AND METHODS: The frequency of bacterial contamination in PCs issued by the Japanese Red Cross was determined using expired PCs before and after the implementation of the diversion method. The culture method was designed such that it yields the least possibility of false-negative results: platelet specimens were sampled after at least 4 days of storage and the inoculum volume was 10 mL for both aerobic and anaerobic bottle cultures.

RESULTS: Of the 21,786 PCs cultured, 36 (0.17%) were confirmed to be bacterially contaminated before the implementation of the diversion method. After its implementation, the number of contaminated PCs decreased to 11 of 21,783 (0.05%) with a reduction rate of 71% and the number of contaminations of clinical importance was 4 (0.018%) excluding PCs positive for *Propionibacterium acnes*. The frequency of contamination by bacteria presumed to originate from donors' blood did not decrease.

CONCLUSION: The effect of the diversion method on the frequency of bacterial contamination is robust. The low incidence of septic reactions after PC transfusion in Japan in spite of the contamination frequency being comparable to those in Western countries and the non-institution of culture screening suggests the importance of a short shelf life (72 hr) for PCs introduced in Japan.

Bacterial contamination of blood components is the major residual infectious risk in modern transfusion medicine in developed countries. The transfusion of blood components with clinically relevant bacterial species at certain concentrations can lead to sepsis or a fatal outcome in transfusion recipients. This is particularly true for platelet concentrate (PC) products that are stored at 20 to 24°C. It is generally accepted that the frequency of bacterial contamination in PCs is approximately 1 in 3000.[1,2] To prevent transfusion-mediated septic reactions, several preventive measures have been proposed or implemented in each step from blood drawing to bedside practice of transfusion, namely, the improvement of the skin disinfection procedure,[3-5] use of a diversion pouch,[6-8] screening by culture for bacteria,[9-14] pH or glucose measurement, screening by amplification of a bacterial genome sequence,[15-17] and use of pathogen reduction/inactivation technologies.[18]

In Japan, all PC products are obtained using the apheresis system and the expiry time for PCs has been limited to 72 hours, which has undoubtedly contributed to the relatively infrequent occurrence of sepsis after PC transfusion. Over the past 8 years, however, two cases of septic reactions, including one fatal case, after PC transfusion have been confirmed by the Japanese Red Cross (JRC) blood center, and the necessity for the implementation of novel strategies for reducing bacterial contamination has been discussed. To this end, it is essential to establish a precise frequency of bacterial contamination in PCs processed under current regulations for blood procurement and processing. JRC systematically cultured more than 20,000 expired PCs using the culture conditions that were expected to provide the lowest possibility of false-negative results. JRC implemented the diversion method in October 2006 in the blood drawing process for PCs and reevaluated thereafter the bacterial contamination frequency in 20,000 expired PCs. In this article, we report the bacterial contamination frequency in PCs before and after the implementation of the diversion method and discuss the possible origin of bacteria detected in PCs obtained from otherwise healthy blood donors in Japan.

ABBREVIATIONS: JRC = Japanese Red Cross; PC(s) = platelet concentrate(s).

From the Japanese Red Cross Tokyo Metropolitan West Blood Center and the Japanese Red Cross Tokyo Metropolitan Blood Center, Tokyo; the Japanese Red Cross Hokkaido Blood Center, Hokkaido; the Japanese Red Cross Miyagi Blood Center, Miyagi; the Japanese Red Cross Aichi Blood Center, Aichi; the Japanese Red Cross Osaka Blood Center, Osaka; the Japanese Red Cross Okayama Blood Center, Okayama; the Japanese Red Cross Kyushu Blood Center, Fukuoka; and the Japanese Red Cross Blood Service Headquarters, Tokyo, Japan.

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MATERIALS AND METHODS

Blood collection

In Japan, six types of PC categorized in accordance with the number of platelets (PLTs) contained have been approved, namely, Units 1, 2, 5, 10, 15, and 20. Unit 5 contains 1.0×10^{11} to 2.0×10^{11} PLTs and the number of PLTs contained in other units is in proportion to the unit number. Units 1 and 2 that are specifically used for infants or neonates, representing only 0.2% of all PCs, have been obtained from whole blood using the buffy coat method. All other units have been procured using the apheresis systems of CCS (Haemonetics, Inc., Tokyo, Japan), Terusys (Terumo, Inc., Tokyo, Japan), and Trima Accel (BCT Japan, Inc., Tokyo, Japan). In 2006, splitting of a larger unit was introduced for processing Units 1 and 2, and all PCs in Japan are now produced using the apheresis system. Unit 10 is the most frequently used, which contains 2.0×10^{11} to 3.0×10^{11} PLTs, representing approximately 80% of all PCs used in Japan.

For skin preparation for venipuncture, the donor's cubital fossa is cleansed by two courses of scrub with an isopropyl alcohol-containing cotton swab (One Shot plus, Hakujuji, Inc., Tokyo, Japan). Povidone-iodine alcohol (Isodine field solution, Meiji, Inc., Tokyo, Japan) is next applied on the area using a cotton-tipped applicator (Sterile Cotton Buds, Kawamoto, Inc., Osaka, Japan) in concentric circles away from the puncture site. After a minimum of 30 seconds of air drying, the donor's vein is punctured and blood is drawn. The diversion method was implemented in PC collection in October 2006 and in whole blood collection early in 2007. Twenty-five milliliters of initial flow of whole blood is collected in the integrated diversion pouch and the diverted blood is used for testing and blood archive registry.

Culture of expired PCs

There are 39 JRC blood centers all over Japan that process and distribute PCs. Among them, seven leading blood centers have laboratories with an automatic blood culture system, BacT/ALERT (Sysmex-bioMérieux, Tokyo, Japan). All PCs expiring in local blood centers after 72 hours of storage were further stored until Day 4 or 5 at 15 to 25°C and then sent to one of the leading laboratories. The PCs were maintained at 2 to 6°C during the transportation. The laboratories started PC culture between Day 4 and Day 9 but mostly did so on Day 5, 6, or 7. Common procedures were determined among the laboratories for sampling, inoculation, culturing, and retesting and were strictly followed by trained staff members; a PLT solution of more than 20 mL was drawn from the PC bag, of which 10 mL was inoculated into an anaerobic culture bottle and the remaining 10 mL into an aerobic bottle (BPN and BPA bottles, respectively, Sysmex-bioMérieux). All the procedures were conducted under aseptic conditions in a laminar air flow hood. The inoculated bottles were kept at 35°C in the BacT/ALERT system, and culture was continued until a positive signal was flagged or continued for 7 days in the absence of a positive signal. When a positive signal was flagged, culture was repeated using the original PC and frozen plasma obtained from the same donation. To confirm the bacterial species, bacteria-positive culture bottles were sent to the central laboratory of Tokyo Red Cross Blood Center and Tokyo Metropolitan Institute of Public Health.

Statistical analysis

The chi-square test was used to compare the bacterial contamination frequency of PCs procured before and after the implementation of the diversion method.

RESULTS

Frequency of bacterial contamination in PCs

During the period from May 2005 through April 2006 before the implementation of the diversion method, 21,786 expired PCs were cultured for bacterial examination. Culture started on Day 4, 5, 6, or 7 for 0.4, 20, 19, and 21% of PCs studied, respectively. There were 57 initial positive cultures, 10 of which were determined to have been caused by an inappropriate positive signal by the culture machine (Table 1). Of the remaining 47, 11 were defined as false-positive cultures on the basis of the negative result of the reculture of the aliquot from the original PC (Table 2). The number of confirmatory positive results

TABLE 1. Frequency of bacterial contamination in PCs collected with or without diversion method*

Variable	Without diversion	With diversion	Reduction (%) by diversion
Number of cultures	21,786	21,783	
Initially positive	57 (0.26)	23 (0.11)	
Machine failure	10	1	
False positive	11	11	
Confirmatory positive	36† (0.17)	11 (0.050)	71 (p = 0.0003)
Anaerobic only‡ (*P. acnes*)	24 (0.11)	7 (0.032)	71 (p = 0.004)
Aerobic and anaerobic§	13 (0.060)	4 (0.018)	70 (p = 0.052)

* Data are reported as number (%) unless otherwise specified.
† One culture was doubly contaminated by *P. acnes* and *Staphylococcus* sp.
‡ All the cultures that were anaerobic bottle positive and aerobic bottle negative were identified to be contaminated by *P. acnes*.
§ All the cultures positive for bacteria using aerobic culture bottle were identified to be also positive using anaerobic culture bottle.

TABLE 2. Bacterial species determined as false positive by repeat negative culture

Without diversion (n = 11)	With diversion (n = 11)
P. acnes (7)*	*P. acnes* (8)†
Bacillus sp. (2)	*Bacillus* sp. (2)
Brevibacillus choshinensis (1)	*Bacillus circulans* (1)
Staphylococcus saccharolyticus (1)	

* The number of cultures initially positive for *P. acnes* was 31.
† The number of cultures initially positive for *P. acnes* was 15.

was 36, representing 0.17% of all PCs cultured. Of these 36, 24 (0.11%) were identified only by anaerobic bottle culture and the bacterial species were all determined to be *Propionibacterium acnes* (Table 1). Thirteen (0.060%) were identified both by aerobic and anaerobic bottle culture. One PC was cocontaminated by *Staphylococcus* sp. and *P. acnes*. Eight non-*P. acnes* bacterial species were considered to be derived from the donors' skin and three from the donors' peripheral blood (Table 3). The remaining two PCs were contaminated by *Staphylococcus aureus* and could be derived either from transient skin flora or the donors' blood stream. *P. acnes* was initially detected in 31 PCs but was not detected in 7 PCs by repeated culture (Tables 2 and 3).

Two months after the introduction of the diversion method, when the staff in every donation site became proficient with the procedure, the culture study of expired PCs restarted in December 2006 and ended with the culture of 21,783 PCs in March 2008. Culture started on Day 4, 5, 6, or 7 for 0, 19, 21, and 24% of PCs studied, respectively. There was no significant difference in the date of start of culture between before and after diversion method. Table 1 shows the results of the study. Twenty-three were initially positive, of which one was caused by machine failure as described previously, 11 were false positive, and 11 were determined as confirmatory positive, representing 0.050% of all cultured PCs. Seven (0.032%) of the 11 PCs were found to be contaminated by *P. acnes* only by anaerobic bottle culture. The remaining four PCs were found to be contaminated by other species both by aerobic and by anaerobic bottle culture, indicating that PCs currently released from JRC blood centers after implementation of the diversion method have a 0.018% frequency of contamination by bacteria other than *P. acnes*. One of the four PCs was positive for *Staphylococcus epidermidis* that could be derived from the donors' skin, two were positive for *Streptococcus dysgalactiae* subsp. *equisimilis* and *Escherichia coli* that could be derived from the donors' peripheral blood, and one was positive for *S. aureus* that could be derived from either origin (Table 3). Our culture study of more than 40,000 expired PCs confirmed that the diversion of initial blood flow into the integrated pouch decreased the contamination rate for all bacterial species by 71% (p = 0.0003 by chi-square test, Table 1).

DISCUSSION

The frequency of bacterial contamination in PCs has been recently studied to evaluate the residual risks in the context of the implementation of culture screening. Most of them report the results obtained from routine culture screening conducted as a release test. There are, however, some limitations in those studies regarding the sensitivity of the culture method used, that is, the limited incubation time before sampling and the limited sample volume inoculated into culture bottles. In this study, the culture procedures employed were designed such that the possibility of false-negative results could be as low as possible: the storage period of PCs at 15 to 25°C before sampling was 4 days at the minimum; PCs were stored at a low temperature after 5 days of storage to prevent autolysis of fully grown bacteria; culture was conducted using anaerobic as well as aerobic bottles; the inoculation volume was 10 mL, which is the maximum volume for each culture bottle; and culture was continued for 7 days. Accordingly, the frequencies of bacterial contamination described in this article would be the highest values obtainable using available techniques for bacterial detection with minimal possibility of false-negative results, although possibility remains that false-negative results occur if bacteria die in PCs with storage.

TABLE 3. Confirmed bacterial species detected in PCs other than *P. acnes*

Estimated origin of bacteria	Without diversion	With diversion
Skin flora	*S. epidermidis* (4)*	*S. epidermidis* (1)
	Staphylococcus sp. (1)	
	CNS† (1)	
	S. saccharolyticus (1)	
	Gram(+) bacillus, nonspore (1)	
Transient skin flora or blood	*S. aureus* (2)	*S. aureus* (1)
Peripheral blood	*S. constellatus* (1)	*Streptococcus dysgalactiae* subsp. *equisimilis* (1)
	Salmonella serovar Choleraesuis (1)	*E. coli* (1)
	E. corrodens (1)	

* Number in parentheses is number of cases.
† CNS = coagulase-negative *Staphylococcus*.

Overall, the implementation of the diversion method significantly reduced the frequency of bacterial contamination in PCs by 71% for all bacterial species (Table 1). If the results obtainable using only aerobic culture bottles are considered, the contamination rates without or with the diversion method was 0.06 and 0.018%, respectively, both of which are the values comparable to those reported in Western countries.[1,9,10,13]

In Japan, approximately 700,000 PCs are released and transfused to patients every year. From the calculation based on the data shown in Table 1, it is estimated that as many as 770 PCs contaminated by *P. acnes* had been released every year from blood centers before the diversion method was implemented. Through the JRC hemovigilance, however, there has been no report of adverse reactions after PC transfusion that implicated the involvement of *P. acnes* contamination. In fact, *P. acnes* has been rarely reported to be of clinical significance in the literature.[18] Possible reasons for these are 1) the clinical virulence of *P. acnes* is usually considered to be very low, 2) PC bags currently used serve the suboptimal culture conditions for *P. acnes* in terms of oxygen delivery, and 3) the growing speed of *P. acnes* in PC bags is very low.[20] In this study, the time required for obtaining the positive signal using BacT/ALERT was 3.14 to 6.83 days (mean, 4.7 days) from the start of culture, indicating that it took a minimum of 7 days from PC donation, far exceeding the shelf life of PCs in Japan. The frequency of contamination with clinical relevance could, therefore, be expressed excluding *P. acnes*-contaminated PCs, namely, 0.018% or 126 products per year after the use of the diversion pouch. The significance of using anaerobic culture bottles here would be that the sample volume is doubled and that it often shows better sensitivity than using aerobic culture bottles.[21]

Jacobs and colleagues[22] calculated the rate of septic reactions after the transfusion of bacterially contaminated components as 41% and the rate of fatality among the septic reactions as 11% on the basis of their elaborate prospective study. With these figures and the contamination rate obtained from this study, the total number of septic reactions and the fatality in Japan are estimated as 52 and 5.7 per year, respectively. However, most of the PCs that Jacobs and colleagues described as contaminated are considered to have had a high bacterial load because the sensitivity of the culture method they used was relatively low (10 colony-forming units/mL) and some PCs that were contaminated were identified while they evaluated only PCs stored for 4 days or more. Moreover, most of the contaminated PCs were transfused 4 and 5 days after donation. Therefore, the rates of septic reactions and fatality described in their study are considered to be the results of the transfusion of PCs, most of which were heavily contaminated. The contaminated PCs in our study could include those that had a low bacterial load if they were transfused within the 3-day shelf life. The estimated frequencies of septic reactions and fatality in Japan that were described previously could, in this context, be overestimated.

After the implementation of the diversion method, the number of contaminated PCs possibly originating from the donors' skin flora except for *P. acnes* decreased markedly from eight to one (p = 0.046, Table 3). On the other hand, the contamination possibly caused by the bacteria from the donors' peripheral blood or transient skin flora remains to be a serious problem, showing a decrease in the number of contaminated PCs from five to three. These observations substantiate the theoretical mechanism of the effect of the method of initial flow diversion.

Through the extensive culture of more than 40,000 expired PCs, we identified several bacterial species that could have caused a serious clinical outcome if PCs contaminated at clinically relevant concentrations were transfused. Both *Streptococcus constellatus* and *Eikenella corrodens* sometimes cause periodontitis, local abscess, sepsis, or meningitis and are frequently found in the oral cavity or upper respiratory tract. JRC experienced a fatal case of sepsis caused by the transfusion of a PC contaminated by *Streptococcus pneumoniae*,[23] which must have been derived from the donor's upper respiratory tract or oral cavity. These observations suggest that the transient bacteremia caused by bacterial invasion into the blood

stream from the oral cavity, periodontal space, or upper respiratory tract is not a rare event but that people with such bacteremia represent a considerable proportion of otherwise healthy blood donors.

Three PCs contaminated by *S. aureus* were identified during the culture study. Through donor interview, it was verified that two of the three had atopic dermatitis on their cubital fossa or face. *S. aureus* has been implicated in bacteremia or sepsis in patients with atopic dermatitis[24,25] and it is possible that the organism invaded the donors' peripheral blood from their skin lesion and eventually contaminated PCs. Skin lesions such as atopic dermatitis may serve as a risk factor for bacteremia in blood donors regardless of whether the lesion is on the venipuncture site or not.

In spite of the considerably high rate of bacterial contamination of PCs, only two septic cases including one fatality have been confirmed over the past 8 years from 2000 through 2007. These figures are smaller than those reported by Eder and coworkers[4] for rate of fatality (1/500,000) and septic reactions (1/75,000) after the implementation of universal culture screening of PCs. The most likely reason for it is that the shelf life of PCs has been limited to 72 hours in Japan. We believe that administration of PCs as soon as possible after processing will remain as the best strategy of preventing transfusion-related sepsis whatever new strategies for the reduction of bacterial contamination in PCs are implemented in the near future, although we detected contamination with *E. coli* and *S. aureus*, two organisms that can grow rapidly in PLT products and lead to life-threatening reactions within 3 days of collection. Another reason may be that all PC products in Japan are obtained using the apheresis system, which would decrease the contamination rate in theory, although some recent articles argue for the similarity of contamination rate for apheresis PLTs and pooled PLTs.[3,26] It is also possible that low sensitivity of the current hemovigilance system contributes to the low rate of septic reactions in Japan. It is, however, highly likely that at least the fatality rate obtained represents a real occurrence, as suggested by Jacobs and coworkers,[22] because it is mandatory in Japan for clinicians to report any serious transfusion-related adverse effect to the Ministry of Health, Labour and Welfare, and every such event is also evaluated by the JRC headquarters.

The introduction of culture screening is unlikely in JRC blood centers because it will oblige us to extend the shelf life to 5 days, which would trade off the real merit of the currently short shelf life of PCs. To determine whether the implementation of a novel strategy such as pathogen inactivation/reduction[27,28] or point-of-issue testing of PCs using a rapid assay[29] is indispensable, it is essential to establish the data for patients to be rescued with the new strategy and the cost required for the institution and maintenance of the new strategy.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to TRANSFUSION.

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