Research article

Presence of *mcr-1*-positive *Enterobacteriaceae* in retail chicken meat but not in humans in the Netherlands since 2009

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Recently, the plasmid-mediated colistin resistance gene *mcr-1* was found in *Enterobacteriaceae* from humans, pigs and retail meat in China. Several reports have documented global presence of the gene in *Enterobacteriaceae* from humans, food animals and food since. We screened several well-characterised strain collections of *Enterobacteriaceae*, obtained from retail chicken meat and hospitalised patients in the Netherlands between 2009 and 2015, for presence of colistin resistance and the *mcr-1* gene. A total of 2,471 *Enterobacteriaceae* isolates, from surveys in retail chicken meat (196 isolates), prevalence surveys in hospitalised patients (1,247 isolates), clinical cultures (813 isolates) and outbreaks in healthcare settings (215 isolates), were analysed. The *mcr-1* gene was identified in three (1.5%) of 196 extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates from retail chicken meat samples in 2009 and 2014. Two isolates were obtained from the same batch of meat samples, most likely representing contamination from a common source. No *mcr-1*-positive isolates were identified among 2,275 human isolates tested. All *mcr*-1-positive isolates were colistin-resistant (minimum inhibitory concentration (MIC) › 2 mg/L). Our findings indicate that *mcr*-1-based colistin-resistance currently poses no threat to healthcare in the Netherlands. They indicate however that continued monitoring of colistin resistance and its underlying mechanisms in humans, livestock and food is needed.

Introduction

The worldwide emergence of extended-spectrum beta-lactamases (ESBL) and carbapenemases has limited the available treatment options for infections with Gram-negative bacteria [1]. Colistin is considered to be an antibiotic of last resort for the treatment of infections with carbapenem-resistant bacteria, and its use in humans is increasing [1].

In November 2015, the presence of a plasmid-mediated colistin-resistance gene, *mcr-1*, in *Enterobacteriaceae* from food animals, food and patients in China was reported [2]. The *mcr-1* gene was detected in 21% of *Escherichia coli* isolates cultured from pigs at slaughter and in 15% of *E. coli* isolates cultured from retail meat between 2011 and 2014. In addition, the *mcr*-1 gene was present in 1.4% of *E. coli* isolates and 0.7% of *Klebsiella pneumoniae* isolates from clinical cultures from patients in two Chinese hospitals in 2014. Directly following this publication, the *mcr-1* gene was reported to be present in 0.2% of ESBL- and AmpC-producing *E. coli* isolates from human bloodstream infections, and in 2% of *E. coli* isolates cultured from imported chicken meat in Denmark since 2012 [3].Hereafter, several reports have documented the global presence of the *mcr-1* gene in *Enterobacteriaceae* cultured from humans, food animals and food [4-13].

Traditionally, colistin resistance was thought to be mediated by chromosomal mutations only, and to spread exclusively via clonal transmission of resistant isolates [14]. The emergence of plasmid-mediated

Table 1

Enterobacteriaceae isolates from retail chicken meat, rectal samples, clinical cultures and outbreaks by year of culture, type of isolate, and colistin-resistance, analysed by whole genome sequencing for the presence of the mcr-1 gene, the Netherlands, 2009–2015 (n = 2,471)

Isolate origin	Year	Type of isolate	Number of isolates	Number of colistin-resistant isolates	Number of *mcr-1*-positive isolates
Retail chicken meat (n = 196)					
Prevalence survey (n = 74)	2009	ESBL-producing *Escherichia coli*	68	NA[a]	1
		ESBL-producing *Klebsiella pneumoniae*	6	NA	0
Prevalence survey (n = 122)	2014	ESBL-producing *E. coli*	119	2	2
		ESBL-producing *K. pneumoniae*	3	0	0
Hospitalised patients, rectal samples (n = 1,247)					
Prevalence survey, 4 hospitals (n = 50)	2009	ESBL-producing *E. coli*	39	NA	0
		ESBL-producing *K. pneumoniae*	11	NA	0
Prevalence surveys, 1 hospital (n = 63)	2013–2014	ESBL-producing *E. coli*	54	0	0
		ESBL-producing *K. pneumoniae*	8	0	0
		ESBL-producing *K. oxytoca*	1	0	0
Prevalence surveys, 14 hospitals (n = 1,134)	2011–2014	ESBL-producing *E. coli*	821	2	0
		ESBL-producing *K. pneumoniae*	172	3	0
		ESBL-producing *K. oxytoca*	13	0	0
		ESBLproducing *Enterobacter cloacae*	77	2	0
		ESBL-producing *Citrobacter* spp.	38	1	0
		ESBL-producing *Morganella morganii*	6	6[b]	0
		Other ESBLproducing *Enterobacteriaceae*	7	0	0
Hospitalised patients, clinical cultures (n = 813)					
Blood cultures, 4 hospitals (n = 25)	2009	ESBL-producing *E. coli*	16	NA	0
		ESBL-producing *K. pneumoniae*	7	NA	0
		ESBL-producing *K. oxytoca*	2	NA	0
Blood cultures, 4 hospitals (n = 77)	2013–2014	ESBL-producing *E. coli*	67[c]	0	0
		ESBL-producing *K. pneumoniae*	8[c]	0	0
		ESBL-producing *K. oxytoca*	2	0	0
Clinical cultures, 14 hospitals (n = 711)	2011–2014	ESBL-producing *E. coli*	546	4	0
		ESBL-producing *K. pneumoniae*	101	2	0
		ESBL-producing *K. oxytoca*	5	0	0
		ESBL-producing *E. cloacae*	46	3	0
		ESBL-producing *Citrobacter* spp.	4	0	0
		ESBL-producing *M. morganii*	3	3[b]	0
		ESBL-producing *Proteus mirabilis*	2	2[b]	0
		ESBL-producing *P. vulgaris* group	1	1[b]	0
		Other ESBL-producing *Enterobacteriaceae*	3	0	0
Outbreaks in healthcare settings (n = 215)					
Several wards, including rehabilitation centre (n = 29)[d]	2012–2015	CTX-M-15 producing *K. pneumoniae*	29	0	0
Surgical ward (n = 14)	2014	*E. cloacae*	14	0	0
Intensive care unit (n = 86)	2009–2014	Colistin-resistant *E. cloacae*	86	86	0
Nursing home (n = 10)	2012	Colistin-resistant KPC-producing *K. pneumoniae*	10	10	0
ERCP related procedures (n = 50)	2014–2015	Colistin-resistant *K. pneumoniae*	50	43	0
Neonatal intensive care unit (n = 26)[d]	2014–2015	Colistin-resistant *Serratia marcescens*	26	26[b]	0

ERCP: endoscopic-retrograde cholangio-pancreaticography; ESBL: extended-spectrum beta-lactamase; KPC: *Klebsiella pneumoniae* carbapenemase; NA: not available.

[a] The *mcr-1*-positive isolate was tested colistin-resistant with Etest.

[b] Intrinsic resistance.

[c] Two *E. coli* isolates and one *K. pneumoniae* isolate were not available for whole genome sequencing.

[d] Outbreak and subsequent surveillance.

Colistin resistance was defined as a colistin minimum inhibitory concentration (MIC) > 2 mg/L, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [26].

Table 2

Characteristics of the *mcr-1*-positive *Escherichia coli* isolates from retail chicken meat, the Netherlands, 2009–2015

Isolate	Origin	Date of purchase	Supermarket	MLST	Serotype	Resistance genes	Plasmid replicons
213	Chicken meat	14 October 2009	A	ST2079	O8:H19	*aadA1, aadA2, aadA3, aph(3')-Ia, aph(3")-Ib, aph(3')-Ic, aph(6)-Id, bla*$_{CTX-M-1}$, *bla*$_{TEM-1B}$, *tet*(A), *mcr-1, lnu*(F), *cmlA1, catA1, sul2, sul3, dfrA5*	FIB, FII, HI2, HI2A, I1, I2, P, po111
14M009386[a]	Chicken meat	29 January 2014	B	ST117	O159:H4	*aadA1, bla*$_{SHV-12}$, *mcr-1, sul1, sul3*	FIB, FII
14M009387[a]	Chicken meat	29 January 2014	B	ST117	O159:H4	*aadA1, bla*$_{SHV-12}$, *mcr-1, sul1, sul3*	FIB, FII

MLST: multilocus sequence typing.

[a] Isolate 14M009386 and 14M009387 were cultured from different meat samples with the same lot number.

colistin resistance enables the much more efficient horizontal transfer of colistin resistance genes to other bacteria, making *mcr-1* a potential threat to public health. The aim of this study was to screen several well-documented strain collections of *Enterobacteriaceae*, obtained from retail chicken meat and hospitalised patients in the Netherlands since 2009, for the presence of colistin resistance and the *mcr-1* gene.

Methods

Strain collections

A total number of 2,471 *Enterobacteriaceae* isolates were analysed for the presence of colistin resistance and the *mcr-1* gene. Isolates originated from prevalence surveys in retail chicken meat (196 isolates), prevalence surveys in hospitalised patients (1,247 isolates), clinical cultures (813 isolates) and several outbreaks in healthcare settings (215 isolates), all collected in the Netherlands between 2009 and 2015.

Retail chicken meat

Two ESBL-producing *Enterobacteriaceae* (ESBL-E) prevalence surveys in Dutch retail chicken meat were performed in 2009 and in 2014 [15,16]. A total number of 196 ESBL-E isolates were obtained, 74 isolates from 71 ESBL-E-positive meat samples in 2009 (89 samples cultured), and 122 isolates from 86 ESBL-E-positive meat samples in 2014 (101 meat samples cultured).

Hospitalised patients, rectal samples

The retail chicken meat surveys in 2009 and 2014 were accompanied by hospital-wide prevalence surveys in patients who were admitted to four hospitals in the region where the chicken meat was bought [15,16]. In 2009, ESBL-E rectal carriage was detected in 45 (5.1%) of 876 patients, who carried 50 ESBL-E isolates. Two repeated prevalence surveys in one of the four hospitals in 2013 and 2014, yielded 63 ESBL-E isolates obtained from 63 (5.9%) ESBL-E carriers among 1,065 patients cultured [17].

A multi-centre cluster-randomised study comparing contact isolation strategies for known ESBL-E carriers was performed in 14 Dutch hospitals between 2011 and 2014 (SoM study) [18]. All consecutive adult patients with a routine clinical culture with ESBL-E were placed on contact precautions and enrolled in the study (=index patient). Ward-based ESBL-E prevalence surveys were performed one week after enrolment of the index patient. Perianal swabs were obtained from 10,691 patients and identified 992 (9.3%) ESBL-E carriers, from whom 1,134 ESBL-E isolates were cultured.

Hospitalised patients, clinical cultures

In 2009, 2013 and 2014, all consecutive ESBL-E isolates from blood cultures were prospectively collected in the four hospitals that participated in the ESBL-E rectal carriage prevalence surveys [15,16]. A total number of 102 ESBL-isolates from blood cultures were obtained, 25 isolates from 23 patients with an ESBL-E-positive blood culture in 2009, and 77 isolates from 76 patients in 2013 and 2014. Three isolates that were collected in 2014 were not available for whole genome sequencing. In the SoM study, a total number of 711 clinical ESBL-E isolates were obtained from 654 ESBL-E-positive patients.

Outbreaks in healthcare settings

Since 2009, several outbreaks with antimicrobial-resistant bacteria in Dutch hospitals and nursing homes have been documented. Six outbreaks, comprising 215 isolates, for which whole genome sequence data were available, were included in this analysis: (i) an outbreak of CTX-M-15-producing *K. pneumoniae* in several wards of a hospital and an associated rehabilitation centre in 2012–2015 (29 isolates) [19]; (ii) an outbreak of *Enterobacter cloacae* in a surgical ward in 2014 (14 isolates); (iii) an outbreak of colistin-resistant *E. cloacae* in an intensive care unit between 2009 and 2014 (86 isolates); (iv) an outbreak of colistin-resistant KPC-producing *K. pneumoniae* in a nursing home in 2012 (10 isolates) [20]; (v) an outbreak of colistin-resistant *K. pneumoniae* in patients after endoscopic retrograde cholangio-pancreaticography (ERCP) procedures in

Table 3

Antimicrobial susceptibility of *mcr-1*-positive *Escherichia coli* isolates from retail chicken meat, the Netherlands, 2009–2015

Antimicrobial agent	Isolate					
	213		14M009386		14M009387	
	MIC (mg/L)	S/I/R	MIC (mg/L)	S/I/R	MIC (mg/L)	S/I/R[a]
Polymyxins						
Colistin	3[b]	R	≥16	R	≥16	R
Penicillins						
Ampicillin	≥32	R	≥32	R	≥32	R
Amoxicillin/clavulanic acid	8	S	≤2	S	4	S
Piperacillin/tazobactam	≤4	S	≤4	S	≤4	S
Cephalosporins						
Cefuroxime	≥64	R	16	R	16	R
Cefotaxime	8	R	4	R	4	R
Ceftazidime	≤1	S	16	R	16	R
Cefepime	2	I	≤1	S	≤1	S
Cefoxitin	≤4	S[c]	≤4	S[c]	≤4	S[c]
Carbapenems						
Meropenem	≤0.25	S	≤0.25	S	≤0.25	S
Imipenem	≤0.25	S	≤0.25	S	≤0.25	S
Aminoglycosides						
Gentamicin	≤1.0	S	≤1	S	≤1	S
Tobramycin	≤1.0	S	≤1	S	≤1	S
Fluoroquinolones						
Ciprofloxacin	0.5	S	≤0.25	S	≤0.25	S
Norfloxacin	2	R	≤0.5	S	≤0.5	S
Folate pathway inhibitors						
Trimethoprim/sulfamethoxazol	≥16/304	R	≤1/19	S	≤1/19	S

I: intermediate; MIC: minimum inhibitory concentration; R: resistant; S: susceptible.

[a] According to the European Committee on Antimicrobial Susceptibility (EUCAST) clinical breakpoints [26].

[b] Etest: MIC = 3 mg/L; Vitek2: MIC = 2 mg/L.

[c] No clinical breakpoint available; S refers to the screening breakpoint for AmpC Enterobacteriaceae.

2014–2015 (50 isolates); and (vi) an outbreak of (intrinsic) colistin-resistant *Serratia marcescens* in a neonatal intensive care unit in 2014–2015 (26 isolates).

Whole genome sequencing and analysis of sequence data

Whole genome sequencing (WGS) was performed, on either a MiSeq, HiSeq 2500 or NextSeq sequencer (Illumina). De novo assembly was performed using CLC genomics Workbench 7.0.4 (Qiagen) or the open source SPAdes 3.5.0 software (http://bioinf.spbau.ru/spades) [21]. Sequence data were screened for the presence of the *mrc-1* gene by running the assembled sequences against a task template containing the *mcr*-1 gene sequence in Ridom SeqSphere+version 3.0.1 (Ridom, Germany) or by uploading the assembled sequences to the open access bioinformatic webtool ResFinder (updated version 2.1, including the *mcr-1* sequence) of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) [22]. For isolates from two outbreaks (colistin-resistant *E. cloacae* and ERCP-related colistin-resistant *K. pneumonia*), the thresholds for sequence identity and coverage length were set to 98% and 60%, respectively, while for all other isolates both thresholds were set to 80%. The sequence data of the *mcr-1*-positive isolates were further analysed by using a genotyping plugin that allowed serotyping of the isolates and detection of acquired antibiotic resistance genes and plasmids with a 80% threshold for both sequence identity and coverage length (BioNumerics v7.6 beta software, Applied Maths). Reference data for acquired antimicrobial resistance genes and plasmid replicons were retrieved from the ResFinder and PlasmidFinder databases (version 9 November 2015) of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) [22,23]. Whole genome multilocus sequence typing (wgMLST) analysis was performed using a pan-genome MLST scheme comprising 9,347 genes, based on 19 well-annotated reference genomes of *E. coli* and *Shigella* spp. (BioNumerics v7.6 beta, Applied Maths). Additionally, single nucleotide polymorphism (SNP) calling was performed by mapping the paired-end reads of isolate 14M009387 and isolate 213 to the de novo assembled genome of isolate 14M009386, using Bowtie 2.5.5 [24] and SAMtools [25]. Resulting Binary Alignment Maps (BAM) files were

Table 4

Whole genome multilocus sequence typing analysis and whole genome single nucleotide polymorphism analysis of *mcr-1*-positive isolates from retail chicken meat, the Netherlands, 2009–2015[a]

Isolate	wgMLST			wgSNP
	Loci shared	Different alleles within shared loci		SNP positions
	n	n	%	n
14M009387	4,243	3	0.07%	8
213	3,791	3,606	95.1%	100,215

MLST: multilocus sequence typing; SNP: single nucleotide polymorphism; wg: whole genome.

[a] Isolate 14M0009386 was used as reference.

used to perform whole genome SNP (wgSNP) analysis (BioNumerics v7.6 beta, Applied Maths).

Antimicrobial susceptibility testing

Isolates for which antimicrobial susceptibility data were available were screened for the presence of colistin resistance. Susceptibility testing of the three *mcr*-1-positive *E. coli* isolates was performed using Vitek2 (bioMérieux, France) and Etest (bioMérieux, France). The breakpoint tables of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used for the interpretation of minimum inhibitory concentrations (MICs) [26]. Isolates with a colistin MIC›2 mg/L were considered colistin-resistant.

Results

An overview of the 2,471 *Enterobacteriaceae* isolates from retail chicken meat, rectal samples, clinical cultures and outbreaks is presented in Table 1. Colistin resistance was found in two (1.6%) of 122 chicken meat-derived ESBL-E isolates, in 14 (1.1%) of 1,247 isolates from ESBL-E rectal carriers, and in 15 (1.8%) of 813 ESBL-E isolates from clinical cultures. The *mcr*-1 gene was detected in three (1.5%) of 196 chicken meat-derived ESBL-producing *E. coli* isolates, one cultured in 2009 and two in 2014. For all three isolates, the *mcr-1* sequence showed 100% similarity to the gene reported in China [2]. None of the 2,275 human isolates harboured the *mcr-1* gene.

Table 2 shows the general and molecular characteristics of the three *mcr-1*-positive *E. coli* isolates. The isolate that was cultured in 2009 had sequence type ST2079, was CTX-M-1-positive and harboured 17 acquired resistance genes. Both isolates from 2014 had sequence type ST117, were SHV-12-positive and harboured five acquired resistance genes. Although these two isolates were cultured from different meat samples of non-Dutch origin, the meat samples had the same lot number and were bought in the same supermarket on the same day. Plasmid replicons were identified in all three isolates, eight in the isolate from 2009 and two in both isolates from 2014. However, none of the plasmid replicons could be linked to the *mcr*-1 gene.

Antimicrobial susceptibilities for the three *mcr*-1-positive *E. coli* isolates are shown in Table 3. All three isolates were colistin-resistant (MIC›2 mg/L). The isolate from 2009 tested colistin-susceptible by Vitek2 (MIC = 2 mg/L), but resistant by Etest (MIC = 3 mg/L). wgMLST analysis showed that the two isolates from 2014 differed by only three (0.07%) of 4,243 shared loci, whereas the isolate from 2009 differed by 3,606 (95.1%) of 3,791 shared loci (Table 4). The two isolates from 2014 differed by only eight SNPs in wgSNP analysis.

Discussion

In our study, covering the period 2009 to 2015, we detected the recently described plasmid-mediated colistin resistance gene, *mcr-1*, in three ESBL-producing *E. coli* isolates from retail chicken meat samples obtained from Dutch supermarkets in 2009 and 2014. All three *mcr*-1-positive isolates were colistin-resistant, and two of them were genetically closely related. No *mcr-1*-positive isolates were detected in a large collection of *Enterobacteriaceae* isolates of human origin that were collected during the same time period and included isolates of four outbreaks with colistin-resistant *Enterobacteriaceae*.

In addition to the recent reports on the global occurrence of the *mcr-1* gene in *Enterobacteriaceae* cultured from humans, food animals and food [2-13], our findings confirm the presence of the *mcr-1* gene in the European setting already since 2009.

The observed 1.5% prevalence of *mcr*-1-positive isolates is comparable with the reported 2% (5/255) prevalence in imported chicken meat in Denmark, and is lower than the 15% (78/523) prevalence in retail meat in China [2,3]. This lower prevalence may be related to the relatively low rates of polymyxin use in livestock in Europe. In 2014, polymyxins constituted only 0.4% (0.34 defined daily dose animal (DDDA)/animal year) of all antibiotics used in broilers in the Netherlands, with a decreasing trend over the last few years [27].

It is noteworthy that the observed 1.5% prevalence of *mcr-1*-positive isolates in ESBL-E isolates from retail chicken meat in this study is similar to the 1.5% phenotypic colistin resistance that was found in *E. coli* isolates cultured from Dutch retail chicken meat in 2014 [27]. Unfortunately, no data are currently available on the resistance mechanisms involved in this phenotypic colistin resistance.

The genetic identity between the two *mcr-1*-positive isolates that were obtained from the same batch of meat samples most likely represents batch contamination from a common source.

The *mcr*-1-positive isolates in this study belong to different sequence types as compared with those that were found to be related to the *mcr*-1 gene in the Chinese and Danish study [2,3]. *E. coli* ST2097 is uncommon in

humans, but has been reported once before in a study on ESBL-producing bacteria in flies from broiler farms in the Netherlands [28]. *E. coli* ST117, on the other hand, is common in both poultry and humans [16,29]. The detection of the *mcr*-1 gene in isolates that belong to different sequence types illustrates the potential for horizontal transfer of this resistance gene.

Although all chicken meat samples were bought in Dutch supermarkets, the labelling of the samples did not provide any clue with respect to the country where animals were raised. Available data on the origin of the chicken meat were limited to the producing country for the samples from 2014 (non-Dutch, European), for the 2009 isolate this information was not available. A non-European origin of the *mcr-1*-positive meat samples can, therefore, neither be confirmed, nor excluded.

The absence of the *mcr*-1 gene in human isolates of various origins is in accordance with observations in previous studies that the presence of the *mcr-1* gene in clinical isolates is still rare. In China, 1.4% (13/902) of clinical *E. coli* isolates and 0.7% (3/420) of clinical *K. pneumoniae* isolates were *mcr-1*-positive, and in Denmark, only 0.2% (1/417) of ESBL- and AmpC-producing *E. coli* isolates from bloodstream infections [2,3]. This absence of the *mcr-1* gene in current Dutch collections of human *Enterobacteriaceae* may in part be due to the low use of colistin and its analogues, the polymyxins, in humans in the Netherlands. In 2014, polymyxins constituted less than 0.1% (0.01 defined daily dose (DDD)/1,000 inhabitant-days) of all systemic antimicrobials used in primary care and ca 0.3% (0.2 DDD/100 patient-days) of systemic antimicrobials used in the hospital setting [30].

Short-read sequence data are not optimal for the assembly of plasmid sequences, which are known to contain multiple repetitive elements. This may explain why the analysis of our sequence data did not reveal a link between the *mcr-1* gene and the plasmid replicons identified.

Although the prevalence of *mcr-1*-positive isolates in meat samples was low, the presence of this colistin resistance gene in food represents a potential public health threat, as it is located on mobile genetic elements that have the potential to spread horizontally to other bacteria. With the increase in carbapenem resistance, the use of colistin is increasing and, herewith, the selective pressure for the spread of *mcr*-1 gene-containing plasmids. As colistin has become one of the last resort antibiotic options to treat severe infections with Gram-negative bacteria, the continued monitoring of colistin resistance and its underlying resistance mechanisms is important, not only in humans, but also in food production animals and food. The emergence of plasmid-mediated colistin resistance underpins the recent proposal of veterinary experts to reconsider the use of colistin and its analogues in food production animals [31].

In conclusion, the plasmid-mediated colistin resistance gene *mcr*-1 was detected in three ESBL-producing *E. coli* isolates that had been cultured from retail chicken meat from Dutch supermarkets in 2009 and 2014. Two isolates were obtained from the same batch of meat samples, which most likely represents contamination from a common source. The *mcr*-1 gene was not present in a large collection of human isolates collected between 2009 and 2015 in the Netherlands. These findings indicate that *mcr*-1-based colistin resistance currently poses no threat to healthcare in the Netherlands, but requires continued monitoring of colistin resistance and its underlying mechanisms in humans, livestock and food.

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Conflict of interest

Katrien De Bruyne is an employee of Applied Maths, a company that develops and sells software for microbiological typing methods. All other authors have no competing interest to declare.

Authors' contributions

MK, MJMB, JR, PH collected the data, MK, MB, JR and KDB performed the molecular analysis, MK, PH, MJMB, MB, KDB, JR, AF, PS and JK participated in drafting the manuscript, MK coordinated and edited the manuscript.

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