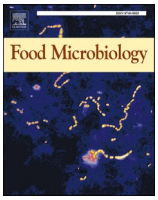
Food Microbiology 96 (2021) 103724

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Isolation, characterization and antibiotic resistance of *Proteus mirabilis* from Belgian broiler carcasses at retail and human stool

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ARTICLE INFO

Keywords:
P. mirabilis
Broiler carcass
Isolation
Characterization
Antibiotic resistance
Human stool

ABSTRACT

Proteus mirabilis is an important pathogen involved in human urinary tract infections, and also more isolated from stools of patients with diarrheal disease than from healthy patients. The role of food, especially poultry products as source for human infection and multi-resistant strains remains unclear. As a resident in broilers' intestines, *P. mirabilis* can contaminate broiler carcasses due to slaughter practices, and be a risk for human infection.

The present study evaluated the performance of five isolation media, and subsequently examined the presence of *P. mirabilis* on broiler carcasses at retail. Additionally, isolates were characterized by the Dienes' test, repetitive element PCR fingerprinting and pulsed-field gel electrophoresis, and their antibiotic resistance profile determined.

Using a combined isolation protocol on blood agar, xylose lysine deoxycholate agar and violet red bile glucose agar, *P. mirabilis* was isolated from 29 out of 80 broiler carcasses (36.25%) with a mean contamination level of 2.25 ± 0.50 $\log_{10}$ CFU/g. A high strain heterogeneity was present in isolates from broilers and human stool. The same strains were not shared, but the antibiotic resistance profiling was similar. A role of poultry products as source for human infection should be taken into account.

1. Introduction

Proteus mirabilis has originally been described and named by Hauser in 1885 (Hoeniger, 1966). Within the family Enterobacteriaceae, the genus *Proteus* is very closely related to the genera *Providencia* and *Morganella*, of which some species came from the re-classification of formerly *Proteus* species. Nowadays, a total of ten species are included in the genus *Proteus*, namely *P. mirabilis*, *P. vulgaris*, *P. penneri, P. terrae, P. hauseri, P. faecis, P. columbea, P. cibi, P. cibarius,* and *P. alimentorum* (https://lpsn.dsmz.de/genus/proteus). *Proteus* species are facultative anaerobic, Gram-negative rods, motile by peritrichous flagella (Manos and Belas, 2006).

Proteus spp. are considered as commensals in the human and other animal intestines, and are widely present in the environment. However, the species *P. mirabilis* is also one of the most common pathogens involved in urinary tract infections in humans, especially in catheter-associated bacteriuria in long-term catheterized patients, as well as significantly more often isolated from stools of patients with diarrheal disease than from healthy patients (Müller, 1989; Sabbuba et al., 2003). Moreover, *P. mirabilis* has been incriminated with nosocomial infections among immunocompromised individuals, including bloodstream infection, cystitis, pyelonephritis and prostatitis, neonatal meningoencephalitis, empyema and diarrheal disease (Jacobsen et al., 2008; Pathirana et al., 2018; Sabbuba et al., 2003).

Proteus mirabilis can be a resident in the intestinal tract of chickens, and consequently potentially be present on poultry products due to fecal contamination at slaughter (Barbour et al., 2012; dos Santos et al., 2014; Wong et al., 2013; Yeh et al., 2018). In addition, *P. mirabilis* is also one of the causative agents of cellulitis in broiler chickens, an inflammation of connective tissues between skin and muscles, and a main cause of condemnation in meat poultry, particularly broiler chickens. It represents another transmission route of *P. mirabilis* on broiler carcass' surfaces at slaughter (Sanches et al., 2020). As such, manipulation and consumption of not well-cooked poultry products are one of the possible

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https://doi.org/10.1016/j.fm.2020.103724
Received 4 August 2020; Received in revised form 27 November 2020; Accepted 22 December 2020
Available online 31 December 2020

transmission routes for human colonization and infection, though this has not been studied in depth (Jacobsen et al., 2008).

At present, no standard method has been developed to isolate *P. mirabilis* from poultry products, and media used in previous studies have not been validated and declared fit for purpose. Furthermore, due to its capacity of swarming on agar plates, determination of the contamination level is complicated.

For epidemiologic purposes, the Dienes' test, repetitive element sequence-based PCR (rep-PCR) and pulsed-field gel electrophoresis (PFGE) have been performed on *P. mirabilis* isolates from patients (Michelim et al., 2008; Pfaller et al., 2000), but very limited on isolates originating from food (Wang et al., 2010). Characterization of isolates both from human and chicken origin could however contribute to the assessment of its potential as foodborne hazard.

The existence of antibiotic resistance amongst *P. mirabilis* isolated from patients and broiler carcasses has previously been reported in Asia and the United states (Kim et al., 2005; Wong et al., 2013), in particular for the classes of β-lactam antibiotics and fluoroquinolones (Boudjemaa et al., 2019; Sanches et al., 2019). The occurrence of antibiotic resistant *P. mirabilis* strains circulating in food animals is an issue of public health relevance. Also in view of clinical treatment in human medicine, antibiotic resistant *P. mirabilis*, especially multi-drug resistant (MDR) strains, is an additional matter of concern. However, comparison of antibiotic resistance profiles between isolates from humans and poultry has not been performed.

Therefore, aims of the present study are to evaluate different isolation media for the isolation of *P. mirabilis*, to assess the occurrence as well as the contamination levels of *P. mirabilis* on broiler carcasses at retail, to assess the genomic variability of isolates form broiler carcasses and human stool, and to construct and compare an antibiotic resistance profile for both groups of isolates.

2. Materials and methods

2.1. Bacterial cultures and isolation media

Three *Proteus mirabilis* reference strains (LMG 15866, 17292 and 17294) were obtained from the BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium. The strains were grown on blood agar (Columbia Agar Base, CM331, OXOID, Basingstoke, UK with 5% defibrinated horse blood (E&O Laboratories Limited, Scotland)), and incubated for 24 h at 30 °C under aerobic condition. Based on several data in literature (Barbour et al., 2012; Wong et al., 2013; dos Santos et al., 2014; Yeh et al., 2018; Yu et al., 2020), five isolation media agar were selected for validation: trypticase soy agar (TSA, CM0131, OXOID), blood agar, MacConkey agar (CM0007, OXOID), xylose lysine deoxycholate agar (XLD, CM0469, OXOID) and violet red bile glucose agar (VRBG, 3564584, BIO-RAD, France).

2.2. Evaluation of isolation procedures from broiler carcasses

From each reference strain, a bacterial suspension was prepared in 10 ml of sterile physiological water with an optical density of 0.9 (measured at 660 nm), which corresponded with a bacterial concentration of approximately 10^9 CFU/ml. Serial 10-fold dilutions in sterile Buffered Peptone Water (BPW, 3564684, Bio-Rad) were prepared.

To assess the recovery, from the expected 10^1–10^3 CFU/ml bacterial suspensions, 10 times, 100 μl were inoculated onto each of the five isolation media. The agar plates were aerobically incubated according to the different protocols in literature: at 30 °C (blood agar and TSA, Yu et al., 2020), or at 37 °C (MacConkey, XLD and VRBG, Barbour et al., 2012; Wong et al., 2013; Yeh et al., 2018), and checked every 3 h for bacterial growth. All colonies were counted, and the colony morphologies recorded.

To evaluate the repeatability of the *P. mirabilis* isolation from broiler skin, 10 g breast skin was taken aseptically from an air packed fresh chicken carcass purchased 4 days before expiration date in a local supermarket in Ghent. The sample was homogenized with 1:10 (W/W) BPW in a sterile stomacher bag for 2 min at 230 rpm using a peristaltic homogenizer (Stomacher® 400 Circulator machine, Seward, UK). To evaluate the natural contamination of *P. mirabilis* and the total aerobic bacteria on the broiler breast skin, 100 μl of a 10-fold dilution series in BPW was brought onto blood agar plates, and incubated aerobically at 30 °C for 24 h. Next, 9 ml of broiler skin homogenate were put in tubes, and one ml of the expected 10^1–10^5 CFU/ml bacterial suspensions per reference strain was added (n = 15: 3 reference strains × 5 dilutions, Supplementary Fig. S1). After vortex mixing for 2 min, 100 μl of each inoculated homogenate was brought onto the withhold isolation media in the recovery test, followed by incubation at 30 or 37 °C up to 72 h, in triplicate. The presence of *P. mirabilis* as well as the total number of other aerobic bacteria were determined. All colonies on each plate were picked and pure-cultured onto TSA plates. Identification was performed using matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS). In brief, one colony of each culture on TSA plate was picked using a sterilized toothpick and smeared gently onto a MALDI-TOF MS target plate (Bruker Daltonics, Bremen, Germany). After air-drying, the sample was covered with 1 μl matrix solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile, deionized water, and trifluoracetic acid (50:47.5:2.5, vv-1). Each series of measurements was preceded by a calibration step with a bacterial test standard (BTS 155 255343; Bruker Daltonics) to validate the run. Mass spectra were generated by a Micro-flex LT MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser (l1⁄4337 nm) operating in linear positive ion detection mode under the Bruker flex Control software (Bruker Daltonics). Identifications were obtained by comparing the mass spectra to the Bruker MSP database (version DB5989) using the Bruker Compass software (Bruker Daltonics) at default settings. Identifications at species or genus level were considered if scores were above 2.0 and 1.7, respectively, according to the report generated by Bruker Compass.

For the evaluation of the reproducibility, all inoculated homogenates were stored for 3 days at 4 °C. Afterwards, 100 μl of each homogenate was brought again onto the withhold isolation media in the recovery test, followed by incubation at 30 °C or 37 °C for up to 48 h, in triplicate. Afterwards, typical colonies on each plate were picked and subcultured on TSA plates and identified by MALDI-TOF MS as described above.

2.3. Isolation of P. mirabilis from broiler carcasses

Eighty fresh air-packed broiler carcasses slaughtered in different slaughterhouses were purchased 2–8 days before expiration date in local supermarkets in Ghent, Belgium in January 2019. Each time, a total of 25 g neck, breast and back skin was taken aseptically, and put into a sterile stomacher bag followed by homogenizing with 1:10 (W/W) BPW for 2 min at 230 rpm using the peristaltic homogenizer mentioned above. Ten-fold serial dilutions of each homogenate (n = 80) were prepared in BPW. Next, 100 μl of each homogenate dilution was brought onto the isolation agar plate with the best performance in the validation test, in duplicate. The plates were incubated at 30 °C or 37 °C aerobically, and growth was checked after 15, 24 and 48 h of incubation for the presence of typical colonies (Fig. 2). Typical colonies were counted and transferred to TSA plates, followed by further identification by MALDI-TOF MS. All confirmed *P. mirabilis* isolates were stored in 80% trypticase soy broth (TSB, CM0129, OXOID)/20% glycerol until further characterization.

2.4. Isolation of P. mirabilis from stool of human patients

To compare *P. mirabilis* strains from human and broiler carcasses, *Proteus mirabilis* isolates from human patient stools were collected. In the period February–March 2019, stool samples from patients, sent to the clinical microbiology laboratory for enteral pathogen investigation,

were examined for the presence of *P. mirabilis*. Isolation was performed according to Leber (2016) using isolation on MacConkey agar. Lactose negative colonies were transferred onto blood agar plates and identified with MALDI-TOF MS. Confirmed *P. mirabilis* isolates were anonymized after registration of a minimal data set on stool consistency and the retrieval of enteral pathogens in the sample. Isolates were frozen at −80 °C until transportation to the research laboratory.

2.5. Characterization of clinical and poultry isolates

The collected *P. mirabilis* isolates from the broiler carcasses were first tested by the Dienes' test and then further characterized by repetitive element PCR fingerprinting (rep-PCR). Additionally, all isolates, including those isolated from human stool, were also typed by pulsed field gel electrophorese (PFGE).

The Dienes' test was performed according to Pfaller et al.(2000). In brief, each time, 3 isolates to be compared were spot inoculated onto the surface of a single blood agar plate. Inoculated plates were incubated at 30 °C for 24 h. The plates were then examined with reflected light for the presence or absence of distinct "Dienes lines" between each pair of isolates. The presence of Dienes lines between two isolates indicated that the isolates were unrelated and thus represented different Dienes types. The lack of Dienes lines indicated that the isolates were indistinguishable and represented the same Dienes type (Pfaller et al., 2000).

All poultry isolates were subsequently typed by rep-PCR. For this, DNA of each isolate was extracted by the method of Pitcher et al. (1989). The primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′), and the PCR conditions previously used by Versalovic et al.(1994) were applied. After fragment size separation in agarose gel electrophoresis, analysis of the rep-PCR fingerprint profiles was performed by BioNumerics v.4.0 software, and dissimilarity metrics were calculated using Pearson correlation coefficient.

Additionally, isolates from broiler carcasses and patients' stools were typed by PFGE. In brief, DNA extraction and plug preparation of the isolates was conducted according to the protocol previously described for *Arcobacter* (Douidah et al., 2014). Digestion was performed with the *SfiI* restriction enzyme for 4 h at 50 °C (Kim et al., 2004). Electrophoresis was carried out with a CHEF DRII (Bio-Rad) through a 1% agarose gel in 0.5 × Tris/borate/EDTA buffer at 14 °C, a voltage of 6 V/cm and a switch angle of 60°, using pulse times ranging from 5 to 50 s for 20 h (Kim et al., 2004). Analysis of the profiles and dissimilarity matrix was performed based on Dice using UPGMA in BioNumerics v.4.0 software. The tolerance rate was determined by the lowest similarity between markers on different gels.

2.6. Antimicrobial susceptibility testing

A representative of each pulsed-type from broilers and human stool was selected for antimicrobial susceptibility testing (AST) using the microbroth dilution technique on an automated system (Phoenix100 Becton Dickinson) according to the manufacturer conditions. The emerge panel NMIC-417 was used for testing, including 27 antimicrobial molecules: amikacin, tobramycin, gentamicin, ampicillin, piperacillin, temocillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, piperacillin/tazobactam, aztreonam, cephalexin, cefuroxime, cefepime, cefixime, cefoxitin, ceftazidime, ceftriaxone, norfloxacin, ciprofloxacin, levofloxacin, colistin, fosfomycin, ertapenem, meropenem, nitrofurantoin, trimethoprim, and trimethoprim/sulfamethoxazole.

Raw data (Minimal inhibitory Concentration in mg/L) were interpreted using EUCAST guidelines Clinical breakpoint for bacteria v 9.0, Jan 2019. Antibiotic molecules for which *P. mirabilis* shows intrinsic resistance according to the EUCAST advice on intrinsic resistance and exceptional phenotypes v 3.2 (February 2020): colistin and nitrofurantoin were not included in the results. For aminoglycosides, the wild type epidemiological cutoff was applied for categorical differentiation of results.

2.7. Data analysis

All counting data were tested for normality and homogeneity of variance using the Shapiro-Wilk test and Levene's test, and then one-way ANOVA was used for general significance. SPSS 20.0.0 was applied for the above statistic analysis. If a significant difference was present, the adjusted significance was calculated between groups based on Benjamini-Hochberg correction with 5% false discovery rate. For the discrimination index (DI) of typing methods, Hunter's generalized formula was used (Hunter and Gaston, 1988). R package "philentropy" (Drost, 2018) and "ape" (Paradis and Schliep, 2019) were used to generate distance matrix of antibiotic files through complete linkage method based on Euclidean distances, and package "ggplot2" (Wickham, 2016) was used for plotting. The category of antimicrobials and MDR definition was based on Magiorakos et al.(2012).

3. Results

3.1. Validation of the isolation procedure from broiler carcasses

All 3 reference strains were recovered on the five isolation media, and the colony morphologies on each plate are shown in Fig. 1A. No significant difference in colony counts was observed between each reference strains and between the different isolation media plates ($p > 0.05$, Table 1A). The determinative swarming ability was only observed on TSA and blood agar for all reference strains. When swarming occurs, individual colony counting through the central points present, is possible around 15 h of incubation on blood agar (Fig. 1B). Longer incubation makes identification of individual colonies almost impossible. Colonies with a black center could be recognized on XLD plates, and pink colored colonies surrounded by a purple halo were present on the VRBG plates. On MacConkey agar, there was no characteristic morphology displayed, and therefore this isolation medium was no further included.

The isolation repeatability on TSA, blood agar, XLD and VRBG was evaluated by the lowest detectable concentration of *P. mirabilis* in inoculated broiler skin (Table 1B). No natural contamination of *P. mirabilis* was detected. On the TSA plates, no typical swarming colonies were observed for any of the 5 inoculated concentrations.

Based on the counting of the bacterial suspensions, the inoculated number in the skin homogenate selected for testing was 2.04, 2.83, and 1.93 $\log_{10}$ CFU/ml for strain LMG 15866, LMG 17292, and LMG 17294, respectively. For strain LMG 15866, the detected concentration was 2.08 $\log_{10}$ CFU/ml on blood agar, 1.95 $\log_{10}$ CFU/ml on VRBG, and 1.00 $\log_{10}$ CFU/ml on XLD. For strain LMG 17292, the detected concentration was 1.07 $\log_{10}$ CFU/ml on blood agar and 1.00 $\log_{10}$ CFU/ml on both VRBG, and XLD. For LMG 17294, the detected concentration was 1.70 $\log_{10}$ CFU/ml on blood agar, 1.30 $\log_{10}$ CFU/ml on VRBG, and 1.60 $\log_{10}$ CFU/ml on XLD.

Concerning the reproducibility, after a 3-day storage at 4 °C, typical *P. mirabilis* colonies were detected on all isolation media plates, except on TSA plates (Table 1C). All 3 reference strains were recovered on XLD plates. Using VRBG, strains LMG 15866 and 17292 were isolated, and only strain LMG 17292 was isolated on blood agar plate. Therefore, XLD, VRBG and blood agar were further included in the next experiment, but TSA plates were no longer withhold.

3.2. Isolation of P. mirabilis on broiler carcasses at retail

Of the 80 broiler carcasses examined, *P. mirabilis* was isolated and confirmed by MALDI-TOF MS from 29 (36%), with a mean contamination level of 2.25 ± 0.50 $\log_{10}$ CFU/g (mean ± SD, Table 2 and Supplementary Table 1). *Proteus mirabilis* was isolated from 15, 14 and 10 carcasses using XLD, blood agar and VBRG isolation medium, respectively. As shown in Supplementary Fig. S2, *P. mirabilis* was isolated by all three media from only two carcasses, and each time from 2 carcasses by

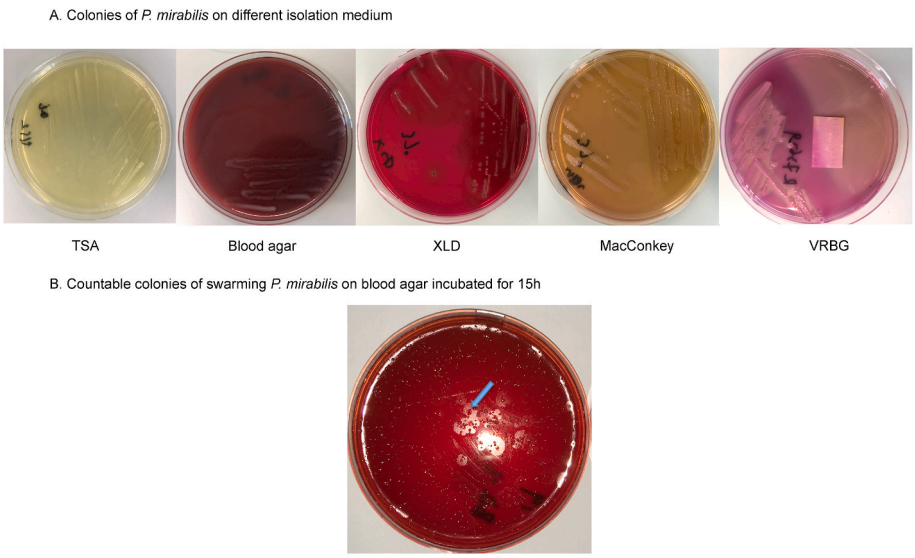

Fig. 1. Morphological presentation of *P. mirabilis* colonies on five different solid isolation agar plates. A: morphological presentation of *P mirabilis* on five different solid isolation media; B: colony count of swarming *P. mirabilis* on blood agar after 15h of incubation.

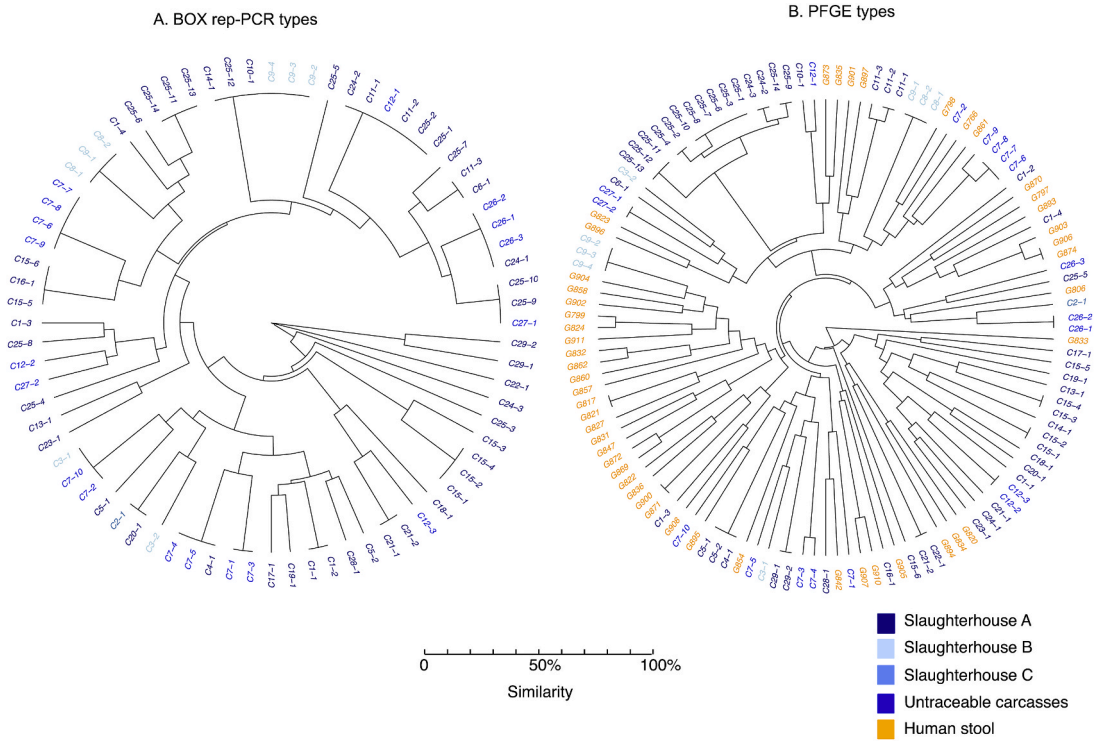

Fig. 2. UPMGA dissimilarity dendrogram of *Proteus mirabilis* isolates characterized by rep-PCR (A, similarity coefficient: DICE) and PFGE (B, similarity coefficient: Pearson correlation). Blue with different shades: broiler carcass isolates from different slaughterhouses; orange: human stool isolates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

combinations of two media. *P. mirabilis* was isolated only by blood agar plate from 8, only by XLD from 9, and only by VBRG from 4 carcasses.

In total, 975 isolates were isolated and further examined, of which 69 isolates (blood agar: n = 34; XLD: n = 32; VRBG: n = 3) displayed the expected typical-morphology on the respective isolation plates. To assess if *P. mirabilis* isolates could be present without displaying a typical colony morphology, 906 non-typical isolates (blood agar: n = 308; XLD: n = 288; VRBG: n = 310) were picked, taking into account thatall different morphologies present on the plates were included, and identified using MALDI-TOF MS. As a result: on blood agar plate, all typical swarming colonies (n = 34) were confirmed as *P. mirabilis*, and none of the 308 non-typical colonies were identified as *P. mirabilis*. Using XLD plates, 29 of the 32 typical colonies were identified as *P. mirabilis*, as well as 3 additional colonies out of the 288 non-typical ones. On VRBG plates, only one of the three typical colonies was confirmed as *P. mirabilis*, but another 9 were identified as *P. mirabilis* from the 310 non-typical colonies. That brought the total number of *P. mirabilis* isolates on 76.

Table 1
Results of the different validation parameters of the isolation procedure (log_{10} CFU/ml) using 5 solid agar media, 3 reference strains and broiler skin homogenates (A: recovery of reference strains from different media; B: the repeatability of reference strains isolation from broiler skin; C: the reproducibility of reference strains isolation from materials after 3-day storage at 4 °C; TSA: trypticase soy agar; BA: blood agar; XLD: xylose lysine deoxycholate agar; VRBG: violet red bile glucose agar; MAC: MacConkey agar).

A. Recovery

Ref	TSA		BA		XLD		MAC		VRBG	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
LMG15866	2.32	±0.06	2.82	±0.05	2.45	±0.12	2.54	±0.16	2.63	±0.14
LMG17292	2.93	±0.11	2.95	±0.03	2.98	±0.08	2.9	±0.05	2.71	±0.15
LMG17294	2	±0.08	2.18	±0.11	1.97	±0.18	2.07	±0.12	1.93	±0.22
Sig.	$p > 0.05$									

B. B. Repeatability* (without MAC)

		Blood agar			TSA			XLD			VRBG		
	Dilution	LMG15866	LMG17292	LMG17294	LMG15866	LMG17292	LMG17294	LMG15866	LMG17292	LMG17294	LMG15866	LMG17292	LMG17294
Before inoculation	0	3.04	3.83	2.93	3	3.79	2.9	2.9	3.72	2.95	2.9	3.62	2.6
Inoculated	10^{-1}	2.08	2.76	1.7	–	–	–	1.7	2.88	1.7	1.95	2.85	1.6
	10^{-2}	–	2.04	–	–	–	–	1	1.7	1.3	1.3	2.15	–
	10^{-3}	–	1.7	–	–	–	–	–	1	–	–	1.78	–
	10^{-4}	–	–	–	–	–	–	–	–	–	–	1	–
	10^{-5}	–	–	–	–	–	–	–	–	–	–	–	–

C. Reproducibility *(without MAC, -: Negative; +: Positive; NA: not determined)

Medium	LMG15866	LMG17292	LMG17294
TSA	–	–	–
BA	–	+	–
XLD	+	+	+
VRBG	+	+	–

Table 2
Occurrence of *P. mirabilis* from broiler carcasses.

	Blood agar	XLD	VRBG	Total
Positivity rate (%)	14/80 (17.5%)	15/80 (18.75%)	10/80 (12.5%)	29/80 (36.25%)
Contamination level (log10 CFU/g)	2.40 ± 0.67	2.28 ± 0.45	2.0 ± 0	2.25 ± 0.50
Typical/Total *P. mirabilis* (n)	34/34	29/32	1/10	64/76

3.3. Characterization of clinical and poultry isolates

The 76 isolates from broiler carcasses were characterized by the Dienes' test and BOX rep-PCR, by which 45 Dienes' types and 41 rep-PCR types were delineated (Fig. 2A). The discrimination index (DI) of Dienes' test was 0.405, as 52 isolates belonged to more than one Dienes' type, whereas the DI of rep-PCR was 0.968, with a marker similarity of 89.7%. Among the 41 rep-PCR types, 31 types were only present on one broiler carcass, while 10 types were present on more than 1 carcass (Fig. 2A). Of these 10 types, 3 types were present on carcasses from the same slaughterhouses and 7 types were present on carcasses from different slaughterhouses.

By PFGE, 52 different pulse-types in the 76 broiler carcass isolates, and 45 in the 48 patient isolates were characterized, setting the ignorance rate at 2% (Fig. 2B). The DI was 0.986. The same pulse-type was not present in both isolate groups. The highest similarity between an isolate from broiler carcasses and patients' stools was 83.3% (two different bands). Two pulse-types were present on more than one carcass, of which one was present on carcasses from the same slaughterhouse and the other was present on carcasses from different slaughterhouses. One pulse type was present on more than one patient.

3.4. Antimicrobial susceptibility

Fifty-two strains from broiler carcasses and 48 from patients' stools, corresponding with the unique pulse-types, were tested for antimicrobial susceptibility. The results are shown in Table 3 and Supplementary Fig. 3. For fluoroquinolones, including ciprofloxacin, norfloxacin and levofloxacin, 48% and 38% of the strains isolated from broiler and human stool of were resistant, respectively. For penicillins, amoxicillin and piperacillin, scored highly resistant with 62% and 44% for broiler and 44% and 23%for human isolates, respectively, while all isolates remained susceptible to temocillin. For penicillins with β-lactamase inhibitors, 21% and 19% of the strains isolated from broiler carcasses and patients were resistant. None of the strains isolated from broiler carcasses was resistant for the antibiotic categories including antipseudomonal penicillins with β-lactamase inhibitors, monobactams, cephamycins, 1st and 2nd generation cephalosporins, 3rd and 4th generation cephalosporins, and carbapenems. Of the strains isolated from human stool, 2% were resistant for antipseudomonal penicillins with β-lactamase inhibitors, and 4% for cephalosporins of both 1st and 2nd generation and 3rd and 4th generation. Strains from patients preserved a 100% susceptibility for monobactams, cephamycins, and carbapenems. For aminoglycosides, all strains retained susceptibility to amikacin, tobramycin proved resistant for 12% and 14% of broiler and human strains respectively, while for gentamicin 44% of the strains from broiler carcasses and 28% strains from patients were not susceptible. To folate pathway inhibitors and phosphonic acids, 40% and 17% of *P. mirabilis* strains from broiler carcass showed resistance, respectively, while 31% and 13% of the strains from patients were resistant.

Considering multi-drug resistant profiles, the definition from Magiorakos et al.(2012) was applied: strains that are resistant for more than three antimicrobial categories are multidrug-resistant (MDR), and the category of antimicrobial were defined. A total of 25 profiles of antibiotic resistance were detected, including one group containing the wild type, which had no acquired resistance for any referred antimicrobial categories (Fig. 3). Among the 24 remaining resistance profiles, 4 included strains resistant for one antimicrobial category, 9 for 2, 5 for 3, 3 for 4, 2 for 5, and 1 for 6 categories (Fig. 3). Hence, in total six MDR profiles were present among these strains. Among these six MDR profiles, four consisted of strains either only isolated from broiler carcasses

Table 3
Results (categorical) of the antimicrobial susceptibility testing of broiler skin and human stool *P. mirabilis* strains (* antibiotic molecules that are not listed in the category for Enterobacteriaceae according to Magiorakos et al., 2012).

Antibiotics		Broiler isolates (n = 52)						Human isolates (n = 48)					
		Susceptible		Intermediate		Resistant		Susceptible		Intermediate		Resistant	
		N	%	N	%	N	%	N	%	N	%	N	%
Fluoroquinolones	ciprofloxacin	27	52%	0	0%	25	48%	30	63%	1	2%	17	35%
	norfloxacin*	27	52%	0	0%	25	48%	31	65%	0	0%	17	35%
	levofloxacin*	27	52%	0	0%	25	48%	30	63%	1	2%	17	35%
Penicillins	ampicillin	20	38%	0	0%	32	62%	27	56%	0	0%	21	44%
	piperacillin*	29	56%	0	0%	23	44%	37	77%	0	0%	11	23%
Special Penicillins	temocillin*	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
Penicillins +β-Lactamase Inhibitors	amoxicillin-clavulanate	41	79%	0	0%	11	21%	39	81%	0	0%	9	19%
Antipseudomonal Penicillins + β-Lactamase Inhibitors	piperacillin-tazobactam	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
	ticarcillin-clavulanate	52	100%	0	0%	0	0%	47	98%	0	0%	1	2%
Monobactams	aztreonam	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
Cephamycins	cefoxitin	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
1st And 2nd Generation Cephalosporins	cephalexin*	52	100%	0	0%	0	0%	46	96%	0	0%	2	4%
	cefuroxime	52	100%	0	0%	0	0%	46	96%	0	0%	2	4%
3rd And 4th Generation Cephalosporins	ceftriaxone	52	100%	0	0%	0	0%	47	98%	0	0%	1	2%
	cefepime	52	100%	0	0%	0	0%	47	98%	0	0%	1	2%
	cefixime*	52	100%	0	0%	0	0%	47	98%	0	0%	1	2%
	ceftazidime	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
Carbapenem	meropenem	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
	ertapenem	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
Aminoglycosides	gentamicin	29	56%	11	21%	12	23%	35	73%	7	15%	6	13%
	tobramycin	46	88%	0	0%	6	12%	41	85%	2	4%	5	10%
Special Aminoglycoside	amikacin	52	100%	0	0%	0	0%	48	100%	0	0%	0	0%
Folate Pathway Inhibitors	trimethoprim-sulfamethoxazole	31	60%	0	0%	21	40%	33	69%	0	0%	15	31%
	trimethoprim*	12	23%	0	0%	40	77%	23	48%	0	0%	25	52%
Phosphonic Acids	fosfomycin w/G6P	43	83%	0	0%	9	17%	42	88%	0	0%	6	13%

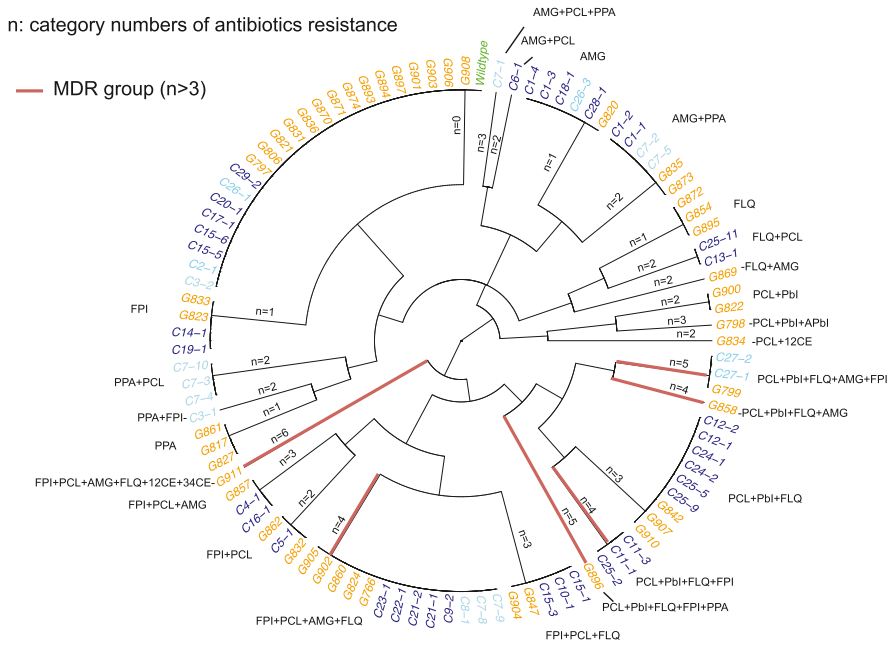

Fig. 3. Antimicrobial resistant profiles clustered using complete linkage method based on Euclidean distances (blue with different shades: broiler carcass isolates from different slaughterhouses; Orange: human stool isolates)*Antibiotic molecules (n = 9) that were not listed or listed as intrinsic resistance in the antibiotics category for Enterobacteriaceae (Magiorakos et al., 2012) were excluded in this analysis, including norfloxacin, levofloxacin, piperacillin, temocillin, cefixime, cephalexin, trimethoprim, colistin, and nitrofurantoin. Also, no isolate was resistant to cefoxitin, meropenem, ertapenem, aztreonam, of which the classes were thus not present in the figure. Abbreviations: AMG-aminoglycosides: gentamicin, tobramycin, amikacin; APbI-Antipseudomonal penicillins with β-lactamase inhibitors: ticarcillin-clavulanic acid and piperacillin-tazobactam; 12CE-1st and 2nd generation cephalosporins: cefuroxime; 34CE-3rd and 4th generation cephalosporins: ceftriaxone, cefepime, ceftazidime; FLQ-Fluoroquinolones: ciprofloxacin; FPI-Folate pathway inhibitors: trimethoprim-sulfamethoxazole; PCL-Penicillins: ampicillin; PbI- Penicillins with β-lactamase inhibitors: amoxicillin-clavulanate; PPA- Phosphonic acids: fosfomycin w/G6P. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(n = 1) or from patients (n = 3). The other two MDR groups contained strains isolated both from broiler carcasses and stool, of which one was resistant for three antimicrobial categories and the other one for four.

4. Discussion

Foodborne pathogens have to be strictly controlled in the food industry, but unlike the common foodborne pathogens associated with poultry products, *Salmonella* and *Campylobacter*, knowledge about emerging pathogens and pathobionts is still very limited. The present study focused on one of those pathobionts, *Proteus mirabilis*, a bacterium that is associated with nosocomial outbreaks of infection (Chow et al., 1979), and who's recent identification as potential pathogen in Crohn's disease recurrences/flare-ups (Hamilton et al., 2018) is under discussion. This bacterium has previously been isolated from broiler carcasses in Asia, but consistent data on its occurrence on European poultry products are lacking (Wong et al., 2013). Hitherto, different isolation and characterization strategies have been applied, hampering the comparison of epidemiological data.

At present, selective media specific for the isolation of *Proteus* spp. are not available. Therefore, an evaluation of isolation media used in previous studies (dos Santos et al., 2014; Kim et al., 2005a,b; Wong et al., 2013, chapter 2) was first performed. On blood agar plates, a typical swarming colony forming was observed, by which the accuracy of *P. mirabilis* isolation showed to be 100%. In contrast, isolation on TSA plates only succeeded when a minimum/critical number of cells were present, as no *P. mirabilis* could be detected when using broiler skin homogenates inoculated with a dilution series of bacterial suspension in the repeatability and reproducibility tests. Media commonly used for the selective isolation of members of the family Enterobacteriaceae, discerning the different genera based on biochemical reactions, were also evaluated. On XLD plates, it has been described that the typical colony morphology of *P. mirabilis* is similar to *Salmonella*: a black colony due to the formation of hydrogen sulfide from thiosulfate, with a white halo (Zajc-Satler and Gragas, 1977). However, in the present study, when pure cultures of the reference strains were cultured, only a few colonies appeared black. This can be explained by the fact that the thiosulfate, key component related to black colony, is not the only nutrition source for *P. mirabilis*, since oxygen and other terminal electron acceptors are also available (Zajc-Satler and Gragas, 1977). This also hampered the preselection of the colonies on the XLD plates when examining the natural contamination of 80 broiler carcasses, as 3 typical colonies turned out not being *P. mirabilis,* and 3 additional colonies of non-typical morphology were identified as *P. mirabilis*. On VRBG agar, a pink colony surrounded by a purple halo due to the dissimilation of glucose, is the typical morphology of *P. mirabilis* (Corry et al., 2003). Although by this morphology, it is difficult to distinguish *P. mirabilis* from other Enterobacteriaceae members, the selectivity of VRBG suppresses a lot of the accompanying bacteria of food, and augments the chance to isolate *P. mirabilis*. Nevertheless, only one of the three typical colonies was confirmed as *P. mirabilis*, and another 9 non-typical were identified as *P. mirabilis*. MacConkey agar, which has previously been used for the isolation of *P. mirabilis* after enrichment (Kim et al., 2005a, b), was excluded due to the lack of typical colony morphology: colonies were not pink, because they are unable to ferment lactose. The same accounts for TSA on which *Proteus mirabilis* was undistinguishable from other bacteria.

Hence, only blood agar, XLD and VRBG were withheld, and as none of the media succeeded to detect all contaminated broiler carcasses, a combined use of these media was necessary.

Results of the examination of 80 broiler carcasses at retail showed for the first time that the mean contamination level with *P. mirabilis* was around 2 $\log_{10}$ CFU per gram skin. In previous studies, an enrichment step was always performed first to isolate *P. mirabilis* from poultry products (Kim et al., 2005; Sanches et al., 2019). In this way, broiler carcasses contaminated with less than 100 CFU/g can be detected, but a quantitative determination of the contamination level is not impossible. On blood agar, after incubation for 15 h, the swarming colonies become discernible and can be counted easily (Fig. 1B). Reading the plates after a standardized incubation time showed to be crucial, with longer incubation, swarming colonies were overgrown by other bacteria. After picking the bacterial growth after 15 h of incubation, no new swarming colonies were detected after longer incubation, suggesting that all typical colonies are present after 15 h. As all typical colonies on blood agar were identified as *P. mirabilis* by MALDI-TOF MS analysis, plating on blood agar is capable to determine the contamination level of *P. mirabilis* on broiler carcasses, if a strict incubation time is applied. Still, not all contaminated carcasses were detected solely with this

medium.

In the present study, 36% of the broiler carcasses at retail were contaminated with *P. mirabilis*. However, this has to be regarded as an underestimation, as false negatives probably occurred. The number of contaminated carcasses in the present study was calculated combining the results from the three selected media, as none of them individually detected all the positive carcasses (Supplementary Fig. S2). Therefore, still no gold standard examination protocol and a single medium for *P. mirabilis* can be proposed. The contamination rate of 36% in the present study, using quantitative analysis, is comparable to other reported rates, in which qualitative analysis was performed: such as 33% in an United States study (Kim et al., 2005a,b), and 23% in a study in Brazil (dos Santos et al., 2014), but might still be an underestimation. A much higher contamination level of 85% has been reported on broiler carcasses in Hong Kong, China (Wong et al., 2013), without an indication of the reason of this high prevalence.

To characterize *P. mirabilis* isolates from broiler carcasses, the Dienes' test, rep-PCR and PFGE were applied. In a previous study, the Dienes' test showed to be powerful in distinguishing clinical isolates, based on the swarming ability of *P. mirabilis*, with a DI of 0.980 (Pfaller et al., 2000). However, results of the present study showed that the Dienes' test had an inferior discriminatory power for *P. mirabilis* on broiler carcasses isolates with a DI of 0.405. Methods with a DI lower than 0.9 are considered unreliable for interpreting typing results (Hunter and Gaston, 1988). Moreover, most isolates presented more than one Dienes' type, again in contrast to the results of the above mentioned study where each clinical isolate represented only one Dienes' type (Pfaller et al., 2000). The mechanism of the Dienes' phenomenon is based on the competition and territoriality of swarming strains, where the self-identity protein IdsD is involved in strain communication (Budding et al., 2009; Saak and Gibbs, 2016). However, details of cell communication, e.g. if different IdsD types exist, are still unclear.

Genomic-based typing, including rep-PCR and PFGE, showed to be reliable in discriminating the broiler carcasses isolates, as well as for clinical isolates based on the results in the present and previous studies (Michelim et al., 2008; Pfaller et al., 2000). The DI of BOX rep-PCR for broiler carcass isolates in the present study (0.968) was slightly lower, though comparable than the one previously reported for isolates from humans (DI = 0.980) by Pfaller et al. (2000). PFGE has long been the gold standard in characterization foodborne pathogens such as *Salmonella, Campylobacter* and *Listeria* (PulseNet, 2016). In the *P. mirabilis* typing tests in the present study, the highest DI (0.986) was obtained with this technique and more strain types could be delineated, both in isolates form broiler carcasses and in isolates from human stool. Both genomic-based typing tests performed equally well in this study, although rep-PCR is faster and easier to perform than PFGE, but the inter-laboratory reproducibility is lower.

Comparison of the different pulse-types learnt that none was present in both groups of test strains. This can however not be interpreted as a proof that there is no zoonotic relation, as one has to take into account that the strains were randomly chosen. Furthermore, even for a foodborne pathogen with a proven zoonotic transmission as *Campylobacter,* for which a world-wide shared pulse-type and multilocus sequence type strain database is in place, due to its genome heterogeneity, the correlation between human infection and the food source has been rarely demonstrated (Barbour et al., 2012; Melo et al., 2015).

One pulse-type from a broiler carcass differed in only two fragments from a pulse-type isolated from stool, indicating they may be derived from the same bacterial lineages or share common evolutionary roots. According to Tenover et al., (1995) , this small difference may be introduced by the deletion of DNA. In a study of extraintestinal pathogenic *Escherichia coli* (ExPEC) isolates from chicken meat and clinical patients, when a maximum of two fragments between an isolate from a patient and one from chicken meat was detected, a relation between both was assumed (Manges, 2016). There was also one identical pulse-type isolated from more than one patient, but no epidemiological link could be demonstrated.

Based on genetic types of *P. mirabilis* isolated from broiler carcasses, a relation linked to slaughterhouses could be demonstrated. Some types were present on different carcasses from the same slaughterhouse (Fig. 2), suggesting a circulation of strains between carcasses at slaughter. Other types were found from carcasses from different slaughterhouses (Fig. 2), suggesting these types circulate between environments, such as rearing farms and transportation vehicles. However, due to the number of the genetic types of *P. mirabilis* is still unknown, the frequency and impact of circulation of *P. mirabilis* between relevant environments are hard to assess.

Resistance to antibiotics, an important issue for both clinical treatment and food safety, has been analyzed for the *P. mirabilis* strains in the present study. Widespread resistance for antibiotic categories of fluoroquinolones and specific broad spectrum β-lactam penicillins in isolates from humans and poultry products has been reported in previous studies (Sanches et al., 2019; Wong et al., 2013). None of the strains from broiler carcasses was resistant to antipseudomonal penicillins with β-lactamase inhibitors, monobactams, temocillin, amikacin, cephamycins, cephalosporins (1st to 4th generation), and carbapenems, whereas some strains from human stool showed resistance to the mentioned above categories of antibiotics. These novel antibiotics have been first applied in human treatment, and are not allowed for use in veterinary medicine with farm animals. After excluding the antibiotics belonging to the intrinsic resistance profile of *P. mirabilis*, i.e. the wild type, resistance for the other antibiotics of both strains from broiler carcasses and patients were acquired post hoc. Antibiotics used in veterinary practice at broiler farms can act as causative triggers for resistance. However, according to the regulation of antibiotic use in veterinary practice in Belgium, except for trimethoprim and sulfonamide, other antibiotic molecules that showed a resistant profile in strains from broiler carcasses should not be used first line (https://www.amcra.be). Higher percentages of isolates resistant against each antibiotic were observed in strains from broiler carcasses than patient, especially for fluoroquinolones. The same trend was also present in the comparison of *P. mirabilis* between human and companion animals (Marques et al., 2019). Several strains isolated from broiler carcass shared the same resistance profile as those from patients, some of which were even MDR, indicating that a circulation of strains or an exchange of antibiotic resistance genes between human and farm animals may exist.

In conclusion, although outbreaks of foodborne *P. mirabilis* infection have been rarely reported yet (Wang et al., 2010), the high occurrence on broiler carcasses suggest a potential risk for human colonization and infection. A need for an overall validated and standardized isolation protocol for *P. mirabilis* is therefore justified, in order to extend the knowledge on its prevalence on food products, and to compare data. If *Proteus mirabilis* deserves the label of established foodborne pathogen, with poultry products as possible source of infection, remains however to be elucidated.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors gratefully acknowledge the technical skills and support of the technicians of the microbiology laboratory in Saint-Lucas Hospital, Ghent, and the contribution of reference strains from Belgian coordinated collections of microorganisms (BCCM).

Funding: this work was supported by the China Scholarship Council (No.201606350041) and a grant from the Special Research Fund (BOF), Ghent University (BOF 17/CHN/057)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2020.103724.

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