Prevalence, Genetic Diversity and Antimicrobial Resistance of Proteus mirabilis Isolated from Dogs Hospitalized in Beijing

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ABSTRACT

Proteus mirabilis is an opportunistic pathogen that causes diarrhea and urinary tract infections in humans and companion animals. Emergence of antimicrobial resistance in P. mirabilis increases risk of failure of antimicrobial therapy in companion animals. The current study determined prevalence, genetic diversity and phenotypic antimicrobial resistance of P. mirabilis isolated from feces of 35 dogs with diarrhea hospitalized in Beijing, China. Random amplified polymorphic DNA (RAPD), pulse-field gel electrophoresis (PFGE), PCR and antimicrobial disk diffusion were used to characterize these isolates. Prevalence of P. mirabilis in feces from hospitalized dogs was 28%. RAPD and PFGE demonstrated a great diversity of isolates. Thirteen (37%) of 35 isolates produced extended-spectrum beta-lactamases (ESBLs), with blatEM being detected in all ESBLs-producing isolates. All isolates were susceptible to imipenem, cefoxitin and cefotaxime/clavulanic. rmtB (with 51% prevalence rate) was the only aminoglycoside 16S rRNA methylase resistance gene identified. Among plasmid-mediated quinolones resistance genes, qnrB (14%) and aac(6’)-Ib (26%) were detected. In conclusion, P. mirabilis isolates from feces of dogs with diarrhea in China had great genetic diversity and a high prevalence produced ESBLs. Consequently, antimicrobial stewardship programs should also target companion animals to reduce emergence of antimicrobial resistance.

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INTRODUCTION

The genus Proteus belongs to the Enterobacteriaceae family. Bacteria in this genus are motile, Gram-negative and can thrive in soil, water and intestinal tracts of mammals (Drzewiecka, 2016). Several species of this genus are known to colonize and infect human and animal hosts, with Proteus mirabilis being the most prevalent one (Wang et al., 2014; Yazdi et al., 2018).

Proteus mirabilis has gained increasing importance as an emerging opportunistic pathogen causing nosocomial infections in humans and animals (Jacobsen and Shirtliff, 2011; Adams-Sapper et al., 2012). In companion animals, they have been implicated as causative agents of urinary tract infections (UTIs) and chronic otitis (Zamankhan Malayeri et al., 2010; Marques et al., 2019). There is supporting evidence that human patients with UTIs caused by P. mirabilis will have the same strain in their stool (Schaffer and Pearson, 2015). Likewise, presence of Proteus spp. in fecal content of dogs is associated with an increased risk of UTIs caused by the same bacteria (Harada et al., 2014). However, whether there is any genetic similarity between isolates that colonize dogs is apparently unknown. Therefore, identification of major clonal complexes colonizing dogs would provide valuable insights into the epidemiology of UTIs and other diseases caused by this important opportunistic pathogen.

In general, infections caused by P. mirabilis are treated with extended-spectrum cephalosporins (ESC), although aminoglycosides or fluoroquinolones are also used. Recently, the emergence of extended-spectrum beta-lactamases (ESBLs) in P. mirabilis has become an
increasing public health concern (Schultz et al., 2017). Whereas it is still uncertain whether and how these bacteria promote dissemination of antimicrobial resistance (AMR), ESBL-producing isolates may be clinically resistant to beta-lactams such as penicillin, aztreonam and cephalosporins (Lin et al., 2019). Currently, prevalence of AMR and associated genes in P. mirabilis isolated from hospitalized dogs in China with diarrhea remains unknown. In this study, we aimed to investigate prevalence, genetic diversity and AMR profile of P. mirabilis isolated from fecal samples of hospitalized dogs with diarrhea. In addition, presence of ESBL-encoding genes in P. mirabilis was also determined.

MATERIALS AND METHODS

Ethics statement: This study was conducted following ethical guidelines of China Agricultural University (CAU), Beijing. Prior to start of the study, ethical approval was granted by the Departmental committee of College of Veterinary Medicine, CAU. Sampling was carried out according to standardized protocols (Jay-Russell et al., 2014) and with prior consent of each owner.

Collection of samples, isolation and identification of Proteus mirabilis isolates: A total of 125 fecal samples was collected aseptically from 125 randomly selected hospitalized dogs with diarrhea at the Small Animal Teaching Hospital, College of Veterinary Medicine, China Agricultural University, from October 2014 to May 2015. A cotton swab was used to inoculate each sample on MacConkey agar (DifcoTM, Becton Dickinson, Sparks, MD USA). Bacterial identification was done based on colony characteristics, triple sugar iron test and gram staining, and isolates were confirmed to be P. mirabilis by API20E biochemical test (BioMerieux, Lyon, France).

DNA extraction and species confirmation: Bacterial genomic DNA was extracted according to manufacturer’s instructions (TIANGEN, Beijing, China). Thereafter, 16S rRNA was amplified by using forward Q-248-F 5'-CGCAAGGATCGACAGTCTCATCA-3' and reverse Q-248-R 5'-CCTGTTAAGTTATC TCTTGAGTGAAT-3' primers (Beijing Sunbiotech Co. Ltd., Beijing, China) with an expected product size of 248 bp (Luo et al., 2008).

RAPD genotyping: For RAPD genotyping, OPX13 5'-ACGGAGCA-3' primers (Beijing Sunbiotech) were used as reported (Michelim et al., 2008). Amplifications were repeated three times (independent DNA extractions and PCR) to evaluate reproducibility for each isolate.

PFGE fingerprinting analysis: Genotyping of P. mirabilis isolates was conducted by macro restriction, using XbaI digestion of bacterial DNA, followed by separation of resulting fragments by PFGE. Resulting maps were prepared using Quantity OneTM software and cluster analysis performed using the UPGMA method based on Dice coefficients, with optimization and tolerance set at 0.5%. Analyses were done in BioNumerics V 5.1 software. Clusters were defined at the 80% similarity level. Degree of relatedness was considered as defined (Tenover et al., 1995).

Antimicrobial susceptibility testing: Antimicrobial susceptibility of all P. mirabilis isolates was done using the disc diffusion method, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (2015). We tested the following antimicrobials: ampicillin (10 µg), ampicillin/sulbactam (10 µg), aztreonam (30 µg), piperacillin (30 µg), mezlocillin (75 µg), imipenem (30 µg), cefotixin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), cefotaxime/sulbactam (30/30 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), ciprofloxacin (0.5 µg) and norfloxacin (10 µg). Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 29213 were used as quality-control strains.

ESBLs phenotyping: All isolates were screened for presence of ESBLs using the disk diffusion method (CLSI, 2015). Briefly, an ESBLs producer was detected with discs containing ceftazidime (CAZ, 30 µg) or cefotaxime (CTX, 30 µg) alone or in combination with clavulanic acid (CLA, 10 µg). Production of ESBLs was confirmed by an increase of ≥5 mm in zones of inhibition of combined disks when compared to agents tested alone.

Detection of ESBLs genes: Primers targeting ESBL genes (blaTEM, blaSHV, blaCTX-M-1, blaOXA1) were synthesized (Beijing Sunbiotech Co., Ltd; Table 1) and reactions done in a final volume of 25 µl of PCR mixture for each sample, following references in Table 1.

Identification of antimicrobial resistance genes: All isolates were screened for presence of AMR genes (Table 2). PCR conditions were used as described (Kim et al., 2005). PCR-positive results were sequenced and compared to existing databases for further confirmation.

Statistical analyses: Confidence intervals (CI) were estimated using q binomial distribution. Fisher’s Exact test was used to compare antimicrobial resistance rates between antimicrobials, and also between ESBL-producers and non-producers. Statistical significance was considered at the 5% level.

RESULTS

Samples collected and prevalence of Proteus mirabilis: A total of 35 Proteus mirabilis were isolated from 125 fecal samples based on colony characteristics, microscopic appearance, biochemical tests and 16S rRNA, with an estimated prevalence of 28% (95% exact confidence interval: 20-37%). Round transparent or translucent colonies with smooth surface and migrating growth at colony edges were visualized on SS agar plate (Fig. 1-A). Colonies were round, smooth and black on HE agar plates (Fig. 1-B). Hydrogen sulfide and gas-producing characteristics of Proteus mirabilis were readily detected in triple sugar iron testing (Fig. 1-C). In addition, when examined with an optical microscope, they were gram negative, polymorphic or rod-shaped bacilli, without spore or capsule formation (Fig. 1-D).
Table 1: Primers used to detect ESBLs resistance genes

| Gene    | Name            | Primer (5’-3’) | Product size (bp) | Reference     |
|---------|-----------------|---------------|------------------|---------------|
| blaTEM | TEM-A           | TCGGTTAGTATCTCCCTGTGG | 297 | Hu, 2007 |
|         | TEM-B           | TTCTGCJTTTGTATGCTCCCT | 593 | Fritsche et al., 2008 |
| blaoxy | OXA-1            | TTTCATAGCTTTCGCTCTTGGT | 520 | Li et al., 2008 |

Table 2: Primers used to identify antimicrobial resistance genes

| Gene   | Sequence (5’-3’) | Product size (bp) | Reference       |
|--------|------------------|------------------|-----------------|
| rmtB   | F: TCAACGATGCCCTCACCTC | 549 | Fritsche et al., 2008 |
|        | R: GCAGGGCCAAAGTTAAATC  | 549 | Fritsche et al., 2008 |
| rmtC   | F: CAGCAAGTACTACAAAGTGG | 752 | Fritsche et al., 2008 |
|        | R: CTCGACGGGCACCAACAG  | 752 | Fritsche et al., 2008 |
| armA   | F: TATGGGCTGTTCATTACCTAGCTATT | 514 | Fritsche et al., 2008 |
|        | R: TCTCCATTCCCTTCTCCTT | 514 | Fritsche et al., 2008 |
| rmtD   | F: ACGTGCGGCTCCATCCATTCCG | 338 | Fritsche et al., 2008 |
|        | R: GCGTGGCGGCTAATCTGTC | 338 | Fritsche et al., 2008 |
| rmtA   | F: TACGGCAGGTATTAGTACGA | 626 | Fritsche et al., 2008 |
|        | R: TTCAAAATCTCAGGCGATGC | 626 | Fritsche et al., 2008 |
| npmA   | F: TGTTGACTGGAGACGGTGAGA | 405 | Fritsche et al., 2008 |
|        | R: CGTCGAAAAATAGCTTTGTC | 405 | Fritsche et al., 2008 |
| qnrA   | F: GATTCTCTACGCGGAT | 578 | Li, 2011 |
|        | R: GTTGCCAGGCAAGAGCTAG | 578 | Li, 2011 |
| qnrB   | F: ATA TTGGCCTG TGG CACTCGTGGTG | 415 | Li, 2011 |
|        | R: TCCCAAGCCTGCTACCTTCTTG | 415 | Li, 2011 |
| qnrS   | F: GATCTCAGGCTCAGGCTCCTG | 521 | Li, 2011 |
|        | R: TGCAGCTTGGCGGGGTAA | 521 | Li, 2011 |
| qnrC   | F: GGGTTGTGTAATTAGTTGA | 307 | Li, 2011 |
|        | R: CACCTACCATATTTTTTTC | 307 | Li, 2011 |
| aac(6’)-I|: F: TTGGAGGCTGCTCATTGCTGCTGCTGCTG | 483 | Park et al., 2006 |
|         | R: GCTCGAATGCTCCCTGCTGGTTT | 483 | Park et al., 2006 |

Table 3: Antimicrobial resistance of 35 Proteus mirabilis isolated from dogs with diarrhea in Beijing

| Antimicrobial | ESBL (%) | non-ESBL (%) | Total (%) |
|---------------|----------|--------------|-----------|
| Ampicillin    | 12 (92)  | 13 (59)      | 25 (71)   |
| Amoxicillin/subbactam | 4 (30) | 4 (18) | 8 (23) |
| Aztreonam     | 1 (8)    | 1 (5)        | 2 (6)     |
| Piperacillin  | 9 (69)   | 6 (27)       | 15 (43)   |
| Mezlocillin   | 12 (92)  | 9 (41)       | 21 (60)   |
| Imipenem      | 0        | 0            | 0         |
| Cefoxitin     | 0        | 0            | 0         |
| Ceftriaxone   | 9 (69)   | 4 (18)       | 13 (37)   |
| Cefotaxime    | 8 (62)   | 5 (23)       | 13 (37)   |
| Cefotaxime/clavulanic | 0 | 0 | 0 |
| Amikacin      | 5 (38)   | 2 (9)        | 7 (20)    |
| Gentamicin    | 6 (46)   | 10 (45)      | 16 (46)   |
| Tobramycin    | 7 (54)   | 11 (50)      | 18 (51)   |
| Tetracycline  | 13 (100) | 22 (100)     | 35 (100)  |
| Chloramphenicol | 8 (62) | 3 (14) | 11 (31) |
| Trimethoprim/sulfamethoxazole | 7 (54) | 17 (77) | 24 (69) |
| Ciprofloxacin | 8 (62)   | 9 (41)       | 17 (49)   |
| Norfloxacin   | 8 (62)   | 5 (33)       | 13 (37)   |

Random amplification of polymorphic DNA (RAPD): Seventeen distinct RAPD profiles were detected (Fig. 2). One profile (RAPD-A) was predominant (composed of 9 isolates). Six isolates were typed as RAPD-B; type RAPD-C and D contained 3 isolates each and 2 isolates were classified as type RAPD-E. The remaining 12 isolates were identified as 12 distinct types (1 isolate per type).

Proteus mirabilis PFGE analysis: Thirty-five P. mirabilis isolates were divided into 9 clusters by PFGE, revealing greater diversity than initially detected using RAPD. From the 9 clusters detected, there were 3 with 3 isolates and 6 with 2 isolates. The remaining 14 isolates were considered unrelated. Isolates from clusters were considered either to be the same strain (all clusters containing 2 isolates and 2 clusters of 3 isolates) or were closely related (2 clusters of 3 isolates) (Fig. 3).

Antimicrobial susceptibility testing: All P. mirabilis isolates were susceptible to cephalin, cefoxitin and cefotaxime/clavulanic, whereas 25 (71%) isolates were susceptible to cepotaxime and ceftriaxone (Table 3). Furthermore, most were resistant to ampicillin (71%), mezlocillin (60%) or trimethoprim/sulfamethoxazole (69%), whereas, resistance to amikacin (20%), amoxicillin/subbactam (23%), piperacillin (43%), gentamicin (46%), tobramycin (51%), chloramphenicol (31%), ciprofloxacin (49%) and norfloxacin (37%) was relatively common. Two (6%) isolates were resistant to aztreonam but none was resistant to imipenem (Table 3).

Table 4: Distribution of 16S rRNA methylases genes and plasmid-mediated quinolone resistance genes in 35 Proteus mirabilis isolated from fecal samples of dogs with diarrhea in Beijing, China

| ESBL types | RAPD types (No. of isolates) | Total (No. of isolates) |
|------------|------------------------------|-------------------------|
| TEM        | 7 (0)                        | 7                       |
| TEM/CTX-M-1 | 0 (0)                       | 0                       |
| TEM/OXA-I  | 2 (1)                       | 4 (1)                   |
| Total      | 9 (2)                        | 13 (2)                  |

Function | Genes |
---------|-------|
16S rRNA methylases | armA, rmtA, mtc, rmtD, nmpA |
Plasmid-mediated quinolone resistance | qnrA, qnrB, qnrC, qnrS |

Thirty (37%) of 35 P. mirabilis isolates were ESBLs-producers. These isolates had decreased susceptibilities to ampicillin, piperacillin, mezlocillin, cefotaxime, ceftriaxone, chloramphenicol and norfloxacin (P<0.05; Table 3).

Detection of potential ESBLs genes: Among the 13 P. mirabilis isolates producing ESBLs, all harbored the blatem gene. Six isolates carried more than 1 ESBLs gene (Table 4). Seven of 9 RAPD-A isolates harbored only the blatem gene whereas the remaining 2 harbored both blatem and blacTX-M-1. Isolates with both blatem and blacOXA-I were detected belonged to RAPD-E (n=2), RAPD-L and RAPD-N types (Table 4).

Detection of genes encoding 16S rRNA methylases: The rmtB gene was detected in 18 (51%) isolates whereas armA, rmtA, rmtC, rmtD and nmpA were not detected in the present study (Table 5).
Detection of plasmid-mediated quinolone resistance genes: Plasmid-mediated quinolone resistance (PMQR) genes qnrB and aac(6’)-Ib were detected in 5 (14%) and 9 (26%) isolates, respectively. No other PMQR gene was detected. All positive isolates were resistant to, at least 1 tested quinolone (norfloxacin and ciprofloxacin) (Table 5).

FIG. 1: Bacterial morphologies of Proteus mirabilis. (A) Growing on SS agar plate. (B) Growing on HE agar plate. (C) Growing in ferric trisaccharide semisolid medium. (D) Gram-stained preparation observed under an optical microscope (1000 x) (D).

FIG. 2: Molecular typing of 35 Proteus mirabilis by RAPD. Isolates were divided into 17 RAPD types (A-Q), with a predominance of type A. Trans-5000 was applied as standard molecular size marker (Trans-Gen).

FIG. 3: Analysis of Pulsed-Field Gel Electrophoresis (PFGE) patterns obtained from the relevant Proteus mirabilis isolates. PFGE profile were compared by BioNumerics V 5.1 sofware. In this experiment, a global reference strain Salmonella enterica serotype Braenderup strain H9812, XbaI digestion, was used as the molecular marker (Marker) (Hunter et al., 2005).

DISCUSSION

To the best of our knowledge, this was the first report of the prevalence, antimicrobial susceptibility and genomic diversity of P. mirabilis isolated from canine fecal samples in China. Prevalence of P. mirabilis in feces from hospitalized diarrheic dogs in Beijing was estimated to be 28%. In the current study, despite the great diversity in P. mirabilis isolated, clustering of isolates was present using either RAPD or PFGE results. Five RAPD types (A, B, C, D and E) containing >1 isolate and indistinguishable isolates were present; consequently, we speculate that there is a common source of P. mirabilis in dogs with diarrhea (e.g. some RAPD types commonly isolated from UTIs and, therefore, being more prevalent in fecal content). Our data also demonstrated that 1 predominant type (RAPD-A) existed among tested isolates. Furthermore, potential ESBLs genes were identified in all type A isolates.

Antimicrobial resistance (AMR) in P. mirabilis isolated from dogs has been reported (Zhang et al., 2018). Broad-spectrum antimicrobials are frequently prescribed for treating bacterial infections in small animals. Overall prevalence of antimicrobial resistance in P. mirabilis isolated from dogs seemed higher than reported for human isolates (Siebor and Newirth, 2013). Prevalence of resistance to ampicillin (71%) was higher than the reported (Harada et al., 2014), probably due to presence of TEM penicillinases (Hordijk et al., 2013). The high prevalence of resistance to quinolone, trimethoprim/sulfamethoxazole and aminoglycosides limits therapeutic options for infections caused by P. mirabilis, as these classes of antimicrobials are frequently used to treat dogs with infections caused by these bacteria (Wong et al., 2015).

ESBLs lead to extensive beta-lactam resistance, undermining efficacious therapeutic options for treating bacterial infections (Schultz et al., 2017). AMR rates differed between ESBL-positive and negative isolates. Among the non-beta-lactam antimicrobials, ESBL isolates also had higher resistance rates to chloramphenicol and some fluoroquinolones. The exact mechanism of increased resistance to non-beta-lactam antimicrobials in ESBL-producing strains remains unknown.

Proteus mirabilis is amongst the most common cephalosporin-resistant bacterial isolates from dogs (Fritsche et al., 2008). In our study, the blaTEM gene was detected in all ESBL-positive isolates. TEM-type ESBLs are among the most prevalent in P. mirabilis isolated from hospitalized patients (Ahn et al., 2017; Rajivgandhi et al., 2018). Despite being commonly present in members of the Enterobacteriaceae family (Alonso et al., 2017), blashV genes were not detected in any strain, corroborating recent results (Zhang et al., 2018). Prevalence of OXA-1 positive isolates in this study was much higher than in a French report (Bonnet et al., 2002).
Aminoglycosides bind irreversibly to the 30S small subunit of bacterial ribosomes, inhibiting mRNA transcription and protein synthesis. Resistance against amikacin (20%) was lower than that of gentamicin (46%) and tobramycin (51%), consistent with previous reports (Wiczkorek et al., 2008). The rmtB gene was detected in 18 P. mirabilis isolates; half of these isolates were resistant to all aminoglycosides evaluated (gentamicin, kanamycin, tobramycin, streptomycin, amikacin). Perhaps high resistance to aminoglycosides was due to presence of the rmtB gene (Fritsche et al., 2008).

qnrB and aac(6’)-Ib are important genes associated with decreased susceptibility to fluoroquinolones. In our study, presence of PMQR genes was always followed by resistance to fluoroquinolones. It is well-established that resistance against fluoroquinolones may also occur due to mutations in the quinolone-resistance determining region of specific genes (de Jong et al., 2018); therefore, mechanisms other than those screened could have been present for fluoroquinolone-resistant P. mirabilis isolates where no resistance genes were detected.

A previous study reported that companion animals and humans could be infected with closely related P. mirabilis strains, suggesting a potential role for companion animals as reservoirs of P. mirabilis to humans (Marques et al., 2019). Therefore, higher frequency of elimination of P. mirabilis to the environment through diarrhea in dogs, combined with high antimicrobial resistance of P. mirabilis in this study, represented a potential threat for human health.

This study had some limitations. Although Proteus spp. can be recovered from healthy individuals, they are more frequently isolated from feces of patients suffering from diarrhea. Whereas it is tempting to assume that P. mirabilis were the causative agents of diarrhea in studied dogs, especially due to their apparent high prevalence, there were not definitively implicated as the cause. Presence of enteropathogenic bacteria causing any intestinal disorder in dogs is clouded by presence of normal indigenous flora. Therefore, in the present study, no causal association can be established between presence of P. mirabilis and diarrhea in dogs. Additionally, as ESBL genes such as blaTEM were not sequenced, we cannot be sure whether variants detected were ESBL-associated genes or not.

Conclusions: P. mirabilis was commonly isolated from diarrheic dogs hospitalized in Beijing. A predominant RAPD type (A) was observed. Clustering of isolates was relatively common; several isolates were either indistinguishable or closely related. Isolates had high resistance to a majority of common antimicrobials. blaTEM gene was detected in all ESBL-producing isolates.

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Authors contribution: PH and JG conceived and designed the study. PH, LL, and SM executed the experiment. PH, ML, SW, DN, NS, HB and BH analyzed the data. We confirm that all authors have interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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