Association among biofilm formation, virulence gene expression, and antibiotic resistance in Proteus mirabilis isolates from diarrhetic animals in northeast China

CURRENT STATUS: UNDER REVIEW

Yadong Sun
Northeast Agricultural University, Liaoning Vocational College of Ecological Engineering
ORCiD: 0000-0001-8959-3364

Shanshan Wen
Northeast Agricultural University

Lili Zhao
Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences

Qiqi Xia
Northeast Agricultural University

Yue Pan
Northeast Agricultural University

Hanghang Liu
Northeast Agricultural University

Chengwei Wei
Northeast Agricultural University

Hongyan Chen
Harbin Veterinary Research Institute

Junwei Ge
Northeast Agricultural University

Hongbin Wang hbwang@neau.edu.cn
Corresponding Author
ORCiD: 0000-0002-6089-7492

DOI: 10.21203/rs.2.16502/v1

SUBJECT AREAS
Animal Science

KEYWORDS
Proteus mirabilis, Animal, Diarrhea, Biofilm, Virulence gene, Antibiotic resistance, Pathogenicity
Abstract

Background The aim of this study was to investigate the association among biofilm formation, virulence gene expression, and antibiotic resistance in P. mirabilis isolates collected from diarrhetic animals (n = 176) in northeast China between September 2014 and October 2016.

Results Approximately 92.05% of the isolates were biofilm producers, whereas 7.95% of the isolates were non-producers. The prevalence of virulence genes in biofilm producers was significantly higher than that in non-producers. Biofilm production was significantly associated with the expression of ureC, zapA, rsmA, hmpA, mrpA, atfA, and pmfA (P < 0.05). Drug susceptibility tests revealed that approximately 76.7% of the isolates were multidrug-resistant (MDR) and extensively drug-resistant (XDR). Biofilm production was significantly associated with resistance to doxycycline, tetracycline, sulfamethoxazole, kanamycin, and cephalothin (P < 0.05). Although the pathogenicity of the biofilm producers was stronger than that of the non-producers, the biofilm-forming ability of the isolates was not significantly associated with morbidity and mortality in mice (P > 0.05).

Conclusion Our findings suggested that a high level of multidrug resistance in diarrhetic animals infected with P. mirabilis in northeast China. The results of this study indicated that the positive rates of the genes expressed by biofilm-producing P. mirabilis isolates were significantly higher than those expressed by non-producing isolates.

1. Background

Proteus mirabilis is a motile gram-negative bacillus belonging to the family Enterobacteriaceae. It is an opportunistic pathogen of great importance that is found in water and soil as well as in the intestinal tracts of mammals. It has been recognized as a leading cause of urinary tract infections [1] and the primary infectious factor in patients
with indwelling urinary catheters [2]. *P. mirabilis* can cause food poisoning, respiratory and wound infections, bacteremia, and other infections [3–6]. In the past decade, diseases associating with *P. mirabilis* infection have also been reported in birds with reproductive failure [7], weaned infant rhesus monkeys (*Macaca mulatta*) and ferrets (*Mustela putorius furo*) with diarrhea [8], and dogs with chronic otitis externa [9]. *P. mirabilis*, as an opportunistic pathogen capable of causing serious infections, should not be neglected. The pathogenicity of *P. mirabilis* is related to various virulence factors, including the presence of fimbriae; flagella-based and swarming motility; urease activity; the presence of specific outer membrane proteins, lipopolysaccharides, and hemolysins; cell invasiveness; and environmental iron binding, most of which are involved in the ability of bacteria to adhere, colonize, and invade tissues, thereby promoting pathogenicity [10, 11]. Virulence gene expression is not the only factor responsible for the pathogenicity of *P. mirabilis*; biofilm formation exacerbates the complexity of *P. mirabilis* infection [12], as biofilms are recognized as the ultimate cause of persistent and destructive infections and inflammatory processes [13]. A biofilm is an assemblage of microbial cells that adhere to specific surfaces and neighboring cells, and it is covered with an extracellular matrix [14, 15]. Biofilms inadvertently contribute to bacterial survival, thereby enabling better adaptation to the conditions of the external environment and more effective use of nutrition [16]. *P. mirabilis* has been found to produce biofilms on a wide range of surfaces, including polystyrene, silicone, latex, glass, and various biological surfaces [17, 18]. In recent years, studies have reported a correlation between biofilm formation and various virulence factors in *P. mirabilis* isolated from humans [19, 20]. For instance, catheter encrustation has been reported to be brought about by the activity of urease-producing biofilms [19, 21]. Jansen et al. discovered that mannose-resistant *Proteus*-like fimbriae produced by *P. mirabilis*, which infects the urinary tract, can induce biofilm formation, with
the fimbriae aiding in the aggregation of the bacteria [20]. However, most reports of \textit{P. mirabilis} describe urinary tract infections in humans, and there are few reports on \textit{P. mirabilis} isolated from diarrhetic animals. In addition, the association between biofilm formation and various virulence factors in \textit{P. mirabilis} isolated from diarrhetic animals is still unknown.

Over the past two decades, due to the identification of multiple multidrug-resistant (MDR) and extensively drug-resistant (XDR) \textit{P. mirabilis} isolates, the treatment of \textit{P. mirabilis} infections has become increasingly difficult [22–25]. The production of biofilms by \textit{P. mirabilis} exacerbates the complexity of bacterial resistance, prolongs the treatment time, and further aggravates the infection. In essence, biofilms protect organisms from the host immune system and antimicrobial agents [26]. Furthermore, a previous study has demonstrated that certain antibiotics can induce biofilm formation [27]. However, it is unclear whether antibiotics are linked to biofilm formation by \textit{P. mirabilis} obtained from diarrhetic animals.

Here, we investigate biofilm formation, antimicrobial susceptibility, and virulence gene expression in \textit{P. mirabilis} recovered from feces of various diarrhetic animals in northeast China and discuss the association among the pathogenicity, drug resistance, and virulence of \textit{P. mirabilis} from the perspective of biofilm formation.

2. Results

2.1 Prevalence of \textit{P. mirabilis} isolates

The prevalence rates of \textit{P. mirabilis} in the diarrheal samples are listed in Table 2. Overall, 28.66% (176/614) of diarrheal specimens were positive for \textit{P. mirabilis}. The positive isolation rate of \textit{P. mirabilis} in different species was less than 40%. The positive rate of \textit{P. mirabilis} isolated from specimens was 32.76% for dog, followed by 28.7% for mink, 23.26% for cattle, and 22.5% for fowl.
2.2 Biofilm formation

Of the *P. mirabilis* isolates (n = 176) tested, 162 (92.05%) were biofilm producers and 14 (7.95%) were non-producers. Of the biofilm producers (n = 162), 78 (48.15%) were moderate biofilm producers, whereas 62 (38.27%) and 22 (13.58%) were strong and weak biofilm producers, respectively.

2.3 Virulence gene expression

The prevalence rates of *ureC*, *zapA*, *rsmA*, *hmpA*, *mrpA*, *atfA*, *pmfA*, *FlIL*, and *ucaA* in *P. mirabilis* are listed in Table 3. Of the *P. mirabilis* isolates, the most prevalent gene was *ureC*, which was identified in 90.91% of the isolates, followed by *zapA*, *rsmA*, *hmpA*, *mrpA*, *atfA*, *pmfA*, *FlIL*, and *ucaA*, which were detected in 85.8%, 81.25%, 70.45%, 65.91%, 64.77%, 60.23%, 56.82%, and 32.95% of the isolates, respectively. The positive rates of the genes identified in biofilm-producing isolates were significantly higher than those in non-producing isolates. Furthermore, the prevalence rates of nine genes in the moderate biofilm producing isolates were significantly higher than those in the strong and weak biofilm producing isolates. All the nine genes tested in this study showed the lowest prevalence rates among the non-producers. Biofilm production was significantly associated with the expression of *ureC*, *zapA*, *rsmA*, *hmpA*, *mrpA*, *atfA*, and *pmfA* (*P* < 0.05).

2.4 Antimicrobial susceptibility testing

The antimicrobial resistance patterns of the *P. mirabilis* isolates are shown in Figure 1. Variable degrees of resistance of these isolates to all antibiotics were observed. The resistance to doxycycline was the highest (112, 63.64%), followed by ampicillin (104, 59.09%), ciprofloxacin (101, 57.39%), streptomycin (98, 55.68%), tetracycline (97, 55.12%), piperacillin/tazobactam (88, 50%), cefotaxime (86, 48.87%), sulfamethoxazole (76, 43.19%), nitrofurantoin (75, 42.61%), polymyxin B (69, 39.2%), ceftriaxone (67,
38.07%), kanamycin (67, 38.07%), ceftazidime (62, 35.23%), gentamicin (60, 34.09%), cephalothin (53, 30.12%), cefoperazone (50, 28.41%), levofloxacin (45, 25.57%), meropenem (44, 25%), and imipenem (36, 20.45%). Of the sensitive strains, meropenem (57.96%) and imipenem (64.78%) showed the strongest antimicrobial effect on *P. mirabilis* (Fig. 1A).

The multi-drug resistant patterns of the *P. mirabilis* isolates are shown in Figure 1B. No isolate was sensitive to all the antibiotics. Of the *P. mirabilis* isolates, 18 and 23 isolates were resistant to only one or two of the 19 antibiotics tested, respectively, and 106 isolates were MDR, whereas 29 isolates were XDR. Thus, approximately 76.7% of the strains exhibited MDR or XDR.

The antibiotic susceptibility patterns of the biofilm producing and non-producing *P. mirabilis* isolates are shown in Table 4. Both biofilm producers and non-producers were highly resistant to doxycycline and moderately resistant to cefotaxime. Of the 19 antibiotics, doxycycline, ampicillin, tetracycline, cefotaxime, and kanamycin were found to be non-susceptible to non-producers. A sensitivity of 62.95% was observed for the biofilm-producing isolates against imipenem, whereas a sensitivity of 85.71% was noticed for the non-producing isolates against meropenem and imipenem. Isolates showing sensitivity to doxycycline, ampicillin, ciprofloxacin, streptomycin, tetracycline, piperacillin/tazobactam, cefotaxime, nitrofurantoin, sulfamethoxazole, and ceftriaxone produced more biofilm than strains showing resistance to these antibiotics. For other antibiotics, such as polymyxin B, ceftazidime, kanamycin, gentamicin, cefoperazone, cephalothin, meropenem, levofloxacin, and imipenem, we observed opposite findings. Biofilm production was significantly associated with the resistance to doxycycline, tetracycline, sulfamethoxazole, kanamycin and cephalothin (*P* < 0.05).

2.5 Pathogenicity test in mice
The morbidity and mortality of mice that received 32 different isolates of pathogenic bacteria is demonstrated in Table 5. Mice in the negative control group were obviously asymptomatic and in good overall health. Within 12 h of challenge, different symptoms of variable degrees were observed in mice of the experimental groups. These symptoms included gloomy spirit, inactivity, and loss of appetite. In the biofilm-producing group, some mice died within 12 h of challenge. Mice in both the biofilm producing and non-producing groups showed severe clinical symptoms, such as diarrhea, abrosis, subdued behavior, hunched appearance, and absence of grooming within 24 h of challenge. Eleven mice (45.83%) in the biofilm producing and non-producing groups died within 48 h of challenge. After 72 h of challenge, the symptoms began to gradually disappear, and the health of most mice returned to normal. Only two mice (25%) in the non-producing group died within 72 h of challenge. The biofilm-forming ability of P. mirabilis was not significantly associated with morbidity and mortality in mice (\( P > 0.05 \)).

3. Discussion

In this study, 176 (28.66%) P. mirabilis isolates were investigated from 614 diarrheal specimens from various animals at different farms in northeast China between September 2014 and October 2016.

Biofilm formation by P. mirabilis has recently become an issue of increasing concern. In a previous study, P. mirabilis isolates recovered from urine samples showed a higher degree of biofilm production than those isolated from different catheter segments [28]. In this study, the prevalence of P. mirabilis was generally high, which is in agreement with studies on urine from catheterized patients. [12, 28]. Taken collectively, these findings indicate that further studies on P. mirabilis biofilm formation are needed to better understand the disease process and to develop new preventive and therapeutic options. As a target gene, ureC, was used to positively identify P. mirabilis as described previously
Ali et al. reported that 96.66% of human P. mirabilis isolates (n = 30) recovered from the urinary tract expressed ureC [29]. In this study, 90.91% of the isolates were positive for ureC. However, its prevalence was relatively low when compared to the results of an earlier study [30]. Our results revealed that only testing ureC genet proved that P. mirabilis may be get negative results. We should used a PCR method based on 16S rRNA expression to detect P. mirabilis. Compared to the results of our study, the prevalence of zapA, rsmA, hmpA, mrpA, atfA, pmfA, FliL, and ucaA was relatively high, however, the overall prevalence of P. mirabilis was much higher in our study than that in previous reports [52, 31–35].

The prevalence rates of nine genes expressed by moderate biofilm producers were significantly higher than those of the same genes expressed by strong and weak biofilm producers. These results show that moderate biofilm producers are highly virulent. This is also the first study reporting that the biofilm-forming ability of P. mirabilis is significantly associated with the expression of ureC, zapA, rsmA, hmpA, mrpA, atfA, and pmfA (P < 0.05). Biofilm formation is associated with the adhesion and aggregation of bacteria [20], and rsmA, mrpA, and atfA have been reported to be involved in bacterial adhesion and aggregation [31–33], which is consistent with the results of this study. Biofilm formation was also associated with ureC expression, as previously reported [19, 21]. However, the association between zapA and hmpA, and biofilm formation needs further study.

In this study, the results of the antimicrobial susceptibility tests revealed high resistance rates of the P. mirabilis isolates to antibiotics, including doxycycline (112, 63.64%), ampicillin (104, 59.09%), ciprofloxacin (101, 57.39%), streptomycin (98, 55.68%), tetracycline (97, 55.12%), and piperacillin/tazobactam (88, 50%). The resistance rates ranged from 30% to 50% for cefotaxime (86, 48.865%), sulfamethoxazole (76, 43.19%), nitrofurantoin (75, 42.61%), polymyxin B (69, 39.2%), ceftriaxone (67, 38.07%),
kanamycin (67, 38.07%), ceftazidime (62, 35.23%), gentamicin (60, 34.09%), and cephalothin (53, 30.11%). The resistant rates were less than 30% for cefoperazone (50, 28.41%), levofloxacin (45, 25.57%), meropenem (44, 25%), and imipenem (36, 20.45%). The *P. mirabilis* isolates that were resistant to ampicillin, streptomycin, ciprofloxacin, cefotaxime, ceftriaxone, and gentamicin were similar to those previously isolated from chicken products in Hong Kong [50]. However, the resistance rates of *P. mirabilis* to streptomycin, sulfamethoxazole, kanamycin, ampicillin, ciprofloxacin, cephalothin, gentamicin, cefotaxime, and ceftazidime were significantly higher than those reported in an earlier study, which isolated *P. mirabilis* from dogs [36]. Our results on the resistance rates of *P. mirabilis* to nitrofurantoin, tetracycline, and polymyxin B were much lower than those reported in a human study [37], although the resistant rates to other antibiotics, except nitrofurantoin, tetracycline, and polymyxin B, were generally high [37,38]. In a previous study, *P. mirabilis* isolates from dogs were found to be highly sensitive to ciprofloxacin and gentamicin [39]. In this study, meropenem and imipenem were the most effective antibiotics against *P. mirabilis*, which is consistent with the results of an earlier study [40]. Meropenem and imipenem are classified as carbapenems, and our results showed that carbapenems were highly effective against *P. mirabilis*. We also found that approximately 76.7% of the isolates were MDR or XDR, which is very high compared to the rate reported in a previous study [41]. This difference in results indicates that resistance to *P. mirabilis* is steadily increasing. It is also important to mention that the high number of MDR *P. mirabilis* isolates obtained from companion animals may pose a potential threat to human health. Further studies are needed to define the mechanism of the resistance, which may improve the treatment of *P. mirabilis* infections in the future. In this study, we found that the resistance to several antibiotics, including piperacillin/tazobactam, ceftazidime, cefoperazone, ceftriaxone, meropenem, and
imipenem, was significantly higher among biofilm producers than non-producers, indicating that biofilm producers were more resistant to antibiotics than non-producers. Presently, few studies have described an association between biofilm formation and drug resistance of *P. mirabilis*, although similar studies have been performed for other pathogens such as uropathogenic *E. coli* [42,43], coagulase-negative staphylococci [44], and *Haemophilus parasuis* [45]. Except for piperacillin/tazobactam, ceftazidime, cefoperazone, ceftriaxone, meropenem, and imipenem, the antibiotic resistance rates of non-producers were higher than those of producers. This finding is consistent with an earlier study that reported non-MDR *Acinetobacter baumannii* isolates to partake in robust biofilm formation [27]. Different resistance mechanisms are likely to be responsible for the differences in antibiotic resistance and biofilm formation in various bacteria. We also found that the biofilm-forming ability of *P. mirabilis* isolates was significantly associated with the resistance to doxycycline, tetracycline, sulfamethoxazole, kanamycin, and cephalothin (*P* < 0.05). β-lactamase has been reported to decrease the ability of *E. coli* to form biofilms by inhibiting peptidoglycans, which are required for the assembly of surface molecules on the biofilm. Under sub-inhibitory concentrations of tetracycline and ampicillin, the overexpression of the TetA(C) pump, which contributes to the osmotic stress response and induces capsular colanic acid production, promoted the formation of mature biofilms [46]. In addition, there was an association between the resistance to aminoglycoside and extracellular DNA in *Pseudomonas aeruginosa* [47]. Thus, we hypothesize that antibiotic resistance is associated with the composition of *P. mirabilis* biofilm, thereby affecting biofilm formation.

Although the pathogenicity of the biofilm producers was stronger than that of the non-producers, the biofilm-forming ability of the isolates was not significantly associated with morbidity and mortality in mice (*P* > 0.05). The high pathogenicity of the biofilm
producers may have been due to the fact that the biofilm-forming ability of *P. mirabilis* associated with the expression of *ureC, zapA, rsmA, hmpA, mrpA, atfA, and pmfA* (*P* < 0.05). Furthermore, it has been reported that *ureC* and *zapA* expressed by *P. mirabilis* associate with diarrhea in goats [48]. Gabidullin et al. found that diarrhea caused by *P. mirabilis* was related to enterotoxins [49]. Thus, the mechanism responsible for *P. mirabilis*-induced diarrhea needs further study.

4. Conclusion

In conclusion, our study revealed a high level of multidrug resistance in diarrhetic animals infected with *P. mirabilis* in northeast China. Therefore, the use of antimicrobial agents in animals needs to be controlled so as to minimize the emergence and eventual spread of resistant pathogens, which is warranted in order to protect human health. The results of this study indicated that the positive rates of the genes expressed by biofilm-producing *P. mirabilis* isolates were significantly higher than those expressed by non-producing isolates. Furthermore, this is the first study to report that the biofilm-forming ability of *P. mirabilis* isolates from diarrhetic animals is significantly associated with the expression of *ureC, zapA, rsmA, hmpA, mrpA, atfA, and pmfA* (*P* < 0.05) and the resistance to doxycycline, tetracycline, sulfamethoxazole, kanamycin, and cephalothin (*P* < 0.05).

Further studies on biofilm formation by *P. mirabilis* are necessary to better understand the disease process and to develop effective treatment for mammals with antibiotic resistant *P. mirabilis*.

5. Methods

5.1 Sample collection

Six hundred and fourteen fecal swabs were collected randomly from different farms in northeast China between September 2014 and October 2016 and transferred to the
laboratory in ice-filled containers. Fecal swabs were collected from different animals (i.e., dog, mink, cattle, and fowl) with diarrhea.

5.2 P. mirabilis screening

*P. mirabilis* was isolated as described previously [50]. To grow the bacteria, the fecal swabs were incubated with 1 mL of Luria–Bertani broth for 6 h at 37 C, and a 30 µL aliquot of the broth was added to buffered peptone water (Aobox, Beijing, China). The resultant mixture was incubated for 24 h at 37 C to enrich the culture, and then streaked onto xylose lysine deoxycholate agar plates (Hopebiol, Qingdao, China). All plates were incubated at 37 C for a minimum of 16 h. One isolate from each sample was selected for further characterization.

5.3 Identification of isolates

To identify *P. mirabilis* isolates, standard biochemical tests were used as described previously [51]. The isolates were gram-negative bacilli that were positive for glucose, methyl red, and urease but negative for maltose, sucrose, mannitol, indole, and gelatin liquefaction. The Voges–Proskauer test yielded negative results for the isolates. Urease C (*ureC*) and mannose-resistant *Proteus*-like fimbriae (*mrpA*) were amplified by the polymerase chain reaction (PCR) to identify *P. mirabilis* from the presumptive isolates [52]. Positive results were randomly selected for sequencing. When the results were negative for *ureC* and *mrpA* expression, a segment of the 16S rRNA gene was amplified. The 16S rRNA segment was sequenced [53]. The sequences were compared with *ureC*, *mrpA*, and 16S rRNA in GenBank using BLAST.

5.4 Quantification of biofilm production

Biofilm production was assessed using a microtiter plate assay as described previously [54]. *P. mirabilis* isolates were grown in tryptic soy broth (Hopebiol) supplemented with 0.25% glucose overnight at 37 C. Subsequently, the cultures were diluted to 1 10^6 CFU
mL\(^{-1}\) in fresh tryptic soy broth supplemented with 0.25% glucose, and 200 µL of the diluted culture was transferred into each well of a sterile flat-bottom 96-well polystyrene microtiter plate. After incubation for 24 h at 37 C, without shaking, the wells were gently washed three times with 200 µL of distilled water. The biofilms were fixed with 200 µL of 99% methanol for 15 min. The supernatant was removed, and the plate was air-dried. The biofilms were then stained with 200 µL of 1% crystal violet for 10 min. The excess dye was removed by washing the plate under running tap water. Finally, the bound crystal violet was released by adding 200 µL of 33% acetic acid. The optical density (OD) of each well was measured at 590 nm using a microtiter plate reader. To determine the background OD, control experiments were performed with uninoculated broth. This assay was repeated three independent times. The biofilm formation capacity of each strain was analyzed using the method of Khoramian et al. [55].

5.5 Expression of virulence genes

P. mirabilis virulence genes were detected by PCR. These genes were ureC, extracellular metalloprotease (zapA), swarming behavior (rsmA), hemolysin (hpmA), mrpA, ambient-temperature fimbriae (atfA), P. mirabilis fimbriae (pmfA), flagellar basal body protein (FlL), and uroepithelial cell adhesin fimbriae (ucaA) (Table 1) [10]. The base sequences and predicted sizes of the PCR products for the specific oligonucleotide primers used in this study are listed in Table 1. Subsequently, total genomic DNA was isolated from stationary-phase broth cultures that were grown overnight in Luria-Bertani broth with the TIANamp Bacterial DNA Kit (TIANGEN, Beijing, China) according to the manufacturer’s instructions. All PCR products were analyzed by agarose gel electrophoresis, and the results were recorded with a gel documentation system. All amplifications were repeated three independent times in parallel with a negative control (with distilled water used as PCR template).
5.6 Antimicrobial resistance test

The antimicrobial resistance of the isolates to 19 antibiotics, including doxycycline, ampicillin, ciprofloxacin, streptomycin, tetracycline, piperacillin/tazobactam, cefotaxime, nitrofurantoin, sulfamethoxazole, ceftriaxone, polymyxin B, ceftazidime, kanamycin, gentamicin, cefoperazone, cephalothin, meropenem, levofloxacin, and imipenem (BIO-KONT, Wenzhou, China), was determined by the Kirby-Bauer disc diffusion method [56]. In brief, 0.5 McFarland *P. mirabilis* inoculum was smeared onto Mueller-Hinton agar plates (Hopebiol), and the antibiotic discs were dispensed on the agar. The plates were incubated at 37°C for 18 h. *Escherichia coli* (*E. coli*) ATCC 25922 was used as the control microorganism. The inhibitory zone around each disc was measured, and the results were interpreted according to the guidelines provided by the manufacturers and the Clinical and Laboratory Standards Institute [57]. The results were interpreted as resistant, intermediate, and susceptible. MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories [58]. Non-MDR was defined as resistance to none or up to two antimicrobial categories [27].

5.7 Pathogenicity test in mice

The pathogenicity test was performed as described previously [59]. In brief, healthy, 6-week-old, female BALB/c mice (*n* = 99) (Changsheng Biotechnology Co., Ltd., Liaoning, China) were used to investigate the pathogenicity of *P. mirabilis*. The animals were randomly divided into thirty-three groups (*n* = 3/per group) as follows: one control group and thirty-two experimental groups, which tested 32 *P. mirabilis* isolates for their ability to produce biofilm, to express various virulence genes, and to resist different drugs. Each mouse received a 0.2-mL intraperitoneal injection of a bacterial suspension at a concentration of 1 \(10^8\) CFU mL\(^{-1}\), whereas control mice were challenged with vehicle
alone. The mice were fed normally and observed daily for the activity level and water intake for up to 14 days, and deaths were recorded.

5.8 Statistical analysis

Data were analyzed with SPSS 18.0 Software (SPSS Statistics, Inc., Chicago, IL, USA). The Chi-square test was adopted for analysis. $P$-values < 0.05 were considered statistically significant.

Abbreviations

$P$. mirabilis, *Proteus mirabilis*; PCR, polymerase chain reaction; MDR, multidrug-resistant; XDR, extensively drug-resistant; BPW, buffered peptone water; OD, optical density; *E. coli*, *Escherichia coli*; AT, annealing temperature.

Declarations

Acknowledgments

The authors thank Dr. Songfeng Zheng (Missouri State University) for his help with statistical analysis. Declarations

Ethics approval and consent to participate

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (2014-SRM-24). The specimens were collected from animals for laboratory analyses only, and unnecessary pain and suffering of the animals was avoided. The animal owners provided written consent for the collection of specimens. Animal experiments were carried out according to animal welfare standards.

Consent for publication

A signed informed consent was obtained from each participant. The informed consent is
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no financial or personal relationships with other individuals or organizations that could have inappropriately influenced or biased the contents of this paper.

Funding

This work was supported by the “Academic Backbone” Project of Northeast Agricultural University (No. 17XG10), the National Natural Science Foundation of China (31672532), and the SIPT Program (201910224139). The funding body had the role of pay the consumption materials used in this study, and had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors’ Contributions

All Authors participated to the study design and planning. Yadong Sun and ShanShan Wen were responsible for the conception and design, acquisition and extraction of data. Yadong Sun carried out the analysis. Yadong Sun, ShanShan Wen, Lili Zhao, Yue Pan and Qiqi Xia were mainly responsible for drafting the manuscript. Yadong Sun, ShanShan Wen, Hanghang Liu, Chengwei Wei, Hongyan Chen, Junwei Ge and Hongbin Wang were involved in interpreting the results, revising the manuscript and gave final approval of the version to be published.

Authors’ Information

Affiliations
College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, P. R. China

Yadong Sun, Shanshan Wen, Qiqi Xia, Yue Pan, Hanghang Liu, Chengwei Wei, Junwei Ge, Hongbin Wang

State Key Laboratory of Veterinary Biotechnology, Heilongjiang Provincial Key Laboratory of Laboratory Animal and Comparative Medicine, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150069, P. R. China

Lili Zhao, Hongyan Chen

Northeastern Science Inspection Station, China Ministry of Agriculture Key Laboratory of Animal Pathogen Biology, Harbin 150030, P. R. China

Junwei Ge

Liaoning Vocational College of Ecological Engineering, Shenyang 110122, P. R. China

Yadong Sun

Corresponding author: Hongbin Wang, E-mail: hbwang@neau.edu.cn

References

[1] Chen CY, Chen YH, Lu PL, Lin WR, Chen TC, Lin CY. Proteus mirabilis urinary tract infection and bacteremia: risk factors, clinical presentation, and outcomes. Journal of Microbiology, Immunology and Infection. 2012;45:228–36.

[2] Liu MC, Lin SB, Chien HF, Wang WB, Yuan YH, Hsueh PR, et al. 10′(Z), 13′(E)-heptadecadienylhydroquinone inhibits swarming and virulence factors and increases polymyxin B susceptibility in Proteus mirabilis. PloS one. 2012;7:e45563.

[3] Mobley HL, Belas R. Swarming and pathogenicity of Proteus mirabilis in the urinary tract. Trends in microbiology. 1995;3:280–4.

[4] Wang Y, Zhang S, Yu J, Zhang H, Yuan Z, Sun Y, et al. An outbreak of Proteus mirabilis food poisoning associated with eating stewed pork balls in brown sauce, Beijing. Food
[5] Wang JT, Chen PC, Chang SC, Shiau YR, Wang HY, Lai JF, et al. Antimicrobial susceptibilities of Proteus mirabilis: a longitudinal nationwide study from the Taiwan surveillance of antimicrobial resistance (TSAR) program. BMC infectious diseases. 2014;14:486.

[6] Habibi M, Karam MRA, Bouzari S. Construction and evaluation of the immune protection of a recombinant divalent protein composed of the MrpA from MR/P fimbriae and flagellin of Proteus mirabilis strain against urinary tract infection. Microbial pathogenesis. 2018.

[7] Cabassi C, Taddei S, Predari G, Galvani G, Ghidini F, Schiano E, et al. Bacteriologic findings in ostrich (Struthio camelus) eggs from farms with reproductive failures. Avian diseases. 2004;48:716–22.

[8] Yu W, He Z, Huang F. Multidrug-Resistant Proteus mirabilis Isolated From Newly Weaned Infant Rhesus Monkeys and Ferrets. Jundishapur journal of microbiology. 2015;8.

[9] Malayeri HZ, Jamshidi S, Salehi TZ. Identification and antimicrobial susceptibility patterns of bacteria causing otitis externa in dogs. Veterinary research communications. 2010;34:435–44.

[10] Rózalski A, Sidorczyk Z, Kotełko K. Potential virulence factors of Proteus bacilli. Microbiology and Molecular Biology Reviews. 1997;61:65–89.

[11] Legnani-Fajardo C, Zunino P, Piccini C, Allen A, Maskell D. Defined mutants of Proteus mirabilis lacking flagella cause ascending urinary tract infection in mice. Microbial pathogenesis. 1996;21:395–405.

[12] Hola V, Peroutkova T, Ruzicka F. Virulence factors in Proteus bacteria from biofilm communities of catheter-associated urinary tract infections. FEMS Immunology & Medical Microbiology. 2012;65:343–9.

[13] Fusco A, Coretti L, Savio V, Buommino E, Lembo F, Donnarumma G. Biofilm Formation
and Immunomodulatory Activity of Proteus mirabilis Clinically Isolated Strains.
International journal of molecular sciences. 2017;18:414.

[14] Donlan RM. Biofilms: microbial life on surfaces. Emerging infectious diseases. 2002;8:881.

[15] Kwiecinska-Pirog J, Skowron K, Bartczak W, Gospodarek-Komkowska E. The ciprofloxacin impact on biofilm formation by Proteus mirabilis and P. vulgaris strains. Jundishapur journal of microbiology. 2016;9.

[16] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284:1318-22.

[17] McLean RJ, Lawrence JR, Korber DR, Caldwell DE. Proteus mirabilis biofilm protection against struvite crystal dissolution and its implications in struvite urolithiasis. The Journal of urology. 1991;146:1138-42.

[18] Rocha SP, Elias WP, Cianciarullo AM, Menezes MA, Nara JM, Piazza RM, et al. Aggregative adherence of uropathogenic Proteus mirabilis to cultured epithelial cells. FEMS Immunology & Medical Microbiology. 2007;51:319-26.

[19] Stickler D, Ganderton L, King J, Nettleton J, Winters C. Proteus mirabilis biofilms and the encrustation of urethral catheters. Urological research. 1993;21:407-11.

[20] Jansen AM, Lockatell V, Johnson DE, Mobley HL. Mannose-resistant Proteus-like fimbriae are produced by most Proteus mirabilis strains infecting the urinary tract, dictate the in vivo localization of bacteria, and contribute to biofilm formation. Infection and immunity. 2004;72:7294–305.

[21] Ranjbar-Omid M, Arzanlou M, Amani M, Shokri Al-Hashem SK, Amir Mozafari N, Peeri Doghaheh H. Allicin from garlic inhibits the biofilm formation and urease activity of Proteus mirabilis in vitro. FEMS microbiology letters. 2015;362:fnv049.

[22] Rahal K, Wang F, Schindler J, Rowe B, Cookson B, Huovinen P, et al. Reports on
surveillance of antimicrobial resistance in individual countries. Clinical infectious diseases. 1997;24:S169-S75.

[23] Alabi OS, Mendonça N, Adeleke OE, da Silva GJ. Molecular screening of antibiotic-resistant determinants among multidrug-resistant clinical isolates of Proteus mirabilis from SouthWest Nigeria. African health sciences. 2017;17:356-65.

[24] Bie L, Wu H, Wang XH, Wang M, Xu H. Identification and characterization of new members of the SXT/R391 family of integrative and conjugative elements (ICEs) in Proteus mirabilis. International journal of antimicrobial agents. 2017;50:242-6.

[25] Bameri Z, Karam MRA, Habibi M, Ehsani P, Bouzari S. Determination immunogenic property of truncated MrpH. FliC as a vaccine candidate against urinary tract infections caused by Proteus mirabilis. Microbial pathogenesis. 2018;114:99-106.

[26] Jacobsen SM, Shirtliff ME. Proteus mirabilis biofilms and catheter-associated urinary tract infections. Virulence. 2011;2:460-5.

[27] Qi L, Li H, Zhang C, Liang B, Li J, Wang L, et al. Relationship between antibiotic resistance, biofilm formation, and biofilm-specific resistance in Acinetobacter baumannii. Frontiers in microbiology. 2016;7.

[28] Kwiecinska-Piróg J, Bogiel T, Skowron K, Wieckowska E, Gospodarek E. Proteus mirabilis biofilm-Qualitative and quantitative colorimetric methods-based evaluation. Brazilian Journal of Microbiology. 2014;45:1423-31.

[29] Ali HH, Yousif MG. Detection of some virulence factors genes of Proteus mirabilis that isolated from urinary tract infection. Int J. 2015;3:156-63.

[30] Zhang W, Niu Z, Yin K, Liu P, Chen L. Quick identification and quantification of Proteus mirabilis by polymerase chain reaction (PCR) assays. Annals of microbiology. 2013;63:683-9.

[31] Shi X, Lin Y, Qiu Y, Li Y, Jiang M, Chen Q, et al. Comparative screening of digestion
tract toxic genes in Proteus mirabilis. PloS one. 2016;11:e0151873.

[32] Barbour EK, Hajj ZG, Hamadeh S, Shaib HA, Farran MT, Araj G, et al. Comparison of phenotypic and virulence genes characteristics in human and chicken isolates of Proteus mirabilis. Pathogens and global health. 2012;106:352–7.

[33] Zunino P, Geymonat L, Allen AG, Legnani-Fajardo C, Maskell DJ. Virulence of a Proteus mirabilis ATF isogenic mutant is not impaired in a mouse model of ascending urinary tract infection. FEMS Immunology & Medical Microbiology. 2000;29:137-43.

[34] Zunino P, Sosa V, Allen AG, Preston A, Schlapp G, Maskell DJ. Proteus mirabilis fimbriae (PMF) are important for both bladder and kidney colonization in mice. Microbiology. 2003;149:3231–7.

[35] Sosa V, Schlapp G, Zunino P. Proteus mirabilis isolates of different origins do not show correlation with virulence attributes and can colonize the urinary tract of mice. Microbiology. 2006;152:2149–57.

[36] Harada K, Niina A, Shimizu T, Mukai Y, Kuwajima K, Miyamoto T, et al. Phenotypic and molecular characterization of antimicrobial resistance in Proteus mirabilis isolates from dogs. Journal of medical microbiology. 2014;63:1561–7.

[37] Adamus-Bialek W, Zajac E, Parniewski P, Kaca W. Comparison of antibiotic resistance patterns in collections of Escherichia coli and Proteus mirabilis uropathogenic strains. Molecular biology reports. 2013;40:3429–35.

[38] Kanayama A, Kobayashi I, Shibuya K. Distribution and antimicrobial susceptibility profile of extended-spectrum β-lactamase-producing Proteus mirabilis strains recently isolated in Japan. International journal of antimicrobial agents. 2015;45:113–8.

[39] Pedersen K, Pedersen K, Jensen H, Finster K, Jensen VF, Heuer OE. Occurrence of antimicrobial resistance in bacteria from diagnostic samples from dogs. Journal of Antimicrobial Chemotherapy. 2007;60:775–81.
[40] Huang Y, Xu Y, Wang Z, Lin X. Antimicrobial resistance and genotype analysis of Extended-Spectrum-β-Lactamase-Producing Proteus mirabilis. Open Journal of Clinical Diagnostics. 2014;4:57.

[41] Okonko I, Nkang A, Fajobi E, Mejeha O, Udeze A, Motayo B, et al. Incidence of multidrug resistant (MDR) organisms in some poultry feeds sold in Calabar Metropolis, Nigeria. Electronic Journal of Environmental, Agricultural & Food Chemistry. 2010;9.

[42] Neupane S, Pant ND, Khatiwada S, Chaudhary R, Banjara MR. Correlation between biofilm formation and resistance toward different commonly used antibiotics along with extended spectrum beta lactamase production in uropathogenic Escherichia coli isolated from the patients suspected of urinary tract infections visiting Shree Birendra Hospital, Chhauni, Kathmandu, Nepal. Antimicrobial resistance and infection control. 2016;5:5.

[43] Tajbakhsh E, Ahmadi P, Abedpour-Dehkordi E, Arbab-Soleimani N, Khamesipour F. Biofilm formation, antimicrobial susceptibility, serogroups and virulence genes of uropathogenic E. coli isolated from clinical samples in Iran. Antimicrobial Resistance & Infection Control. 2016;5:11.

[44] Shrestha LB, Bhattarai NR, Khanal B. Antibiotic resistance and biofilm formation among coagulase-negative staphylococci isolated from clinical samples at a tertiary care hospital of eastern Nepal. Antimicrobial Resistance & Infection Control. 2017;6:89.

[45] Zhang J, Xu C, Shen H, Li J, Guo L, Cao G, et al. Biofilm formation in Haemophilus parasuis: relationship with antibiotic resistance, serotype and genetic typing. Research in veterinary science. 2014;97:171-5.

[46] May T, Ito A, Okabe S. Induction of multidrug resistance mechanism in Escherichia coli biofilms by interplay between tetracycline and ampicillin resistance genes. Antimicrobial agents and chemotherapy. 2009;53:4628-39.

[47] Hall CW, Mah TF. Molecular mechanisms of biofilm-based antibiotic resistance and
tolerance in pathogenic bacteria. FEMS Microbiology Reviews. 2017;41:276-301.

[48] Yue L. Identification of Proteus Mirabilis from Goat and Detection of its Major Virulence Genes Southwest University for Nationalities 2016.

[49] Gabidullin Z, Zhukova S, Ezepchuk I, Bondarenko V. The detection of a choleriform thermolabile enterotoxin in clinical strains of Proteus isolated in different infections. Zhurnal mikrobiologii, epidemiologii, i immunobiologii. 1989:14–6.

[50] Wong MHY, Wan HY, Chen S. Characterization of multidrug-resistant Proteus mirabilis isolated from chicken carcasses. Foodborne pathogens and disease. 2013;10:177–81.

[51] Songer J, Post K. Veterinary Microbiology: bacterial and fungal agents of animal disease. St Louis. Elsevier Saunders, St Louis. 2005.p.687.

[52] Stankowska D, Kwinkowski M, Kaca W. Quantification of Proteus mirabilis virulence factors and modulation by acylated homoserine lactones. J Microbiol Immunol Infect. 2008;41:243–53.

[53] Leite A, Miguel M, Peixoto R, Ruas-Madiedo P, Paschoalin V, Mayo B, et al. Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains. Journal of dairy science. 2015;98:3622–32.

[54] Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. Journal of microbiological methods. 2008;72:157-65.

[55] Khoramian B, Jabalameli F, Niasari-Naslaji A, Taherikalani M, Emaneini M. Comparison of virulence factors and biofilm formation among Staphylococcus aureus strains isolated from human and bovine infections. Microbial pathogenesis. 2015;88:73–7.

[56] Bauer A, Kirby W, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American journal of clinical pathology. 1966;45:493.

[57] Wayne P. CLSI. Performance Standards for Antimicrobial Susceptibility Testing;
Twenty-Fifth Informational Supplement. CLSI Document M100-S25, Clinical and Laboratory Standards Institute. 2015.

[58] Magiorakos AP, Srinivasan A, Carey R, Carmeli Y, Falagas M, Giske C, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection. 2012;18:268–81.

[59] Osman KM, Samir A, Abo-Shama UH, Mohamed EH, Orabi A, Zolnikov T. Determination of virulence and antibiotic resistance pattern of biofilm producing Listeria species isolated from retail raw milk. BMC microbiology. 2016;16:263.

Tables

Table 1. Primers used in the PCRs carried out in this study.
| Target gene | Primer | Nucleotide Sequence (5’-3’) | Amplicon (bp) | AT* | Reference |
|-------------|--------|-----------------------------|---------------|-----|-----------|
| 16S rRNA    | 27F    | AGAGTTTGATC CTGGCTCAG GGTATACCTGTT ACGACCTT | 1463 | 49 | Leite et al. (2015) |
|             | 1492R  |                             |               |     |           |
| ureC        | ureC-F | GTTATTTGTGAT GTTATGGG ATAAGGGTGTT TACGCCAGA | 317 | 52 |           |
|             | ureC-R |                             |               |     |           |
| zapA        | zapA-F | ACCGCAAGGAAA ACATATAGCCCC GGCACATATCT CGGAAATACATTCA | 540 | 53 | Stankowska et al. (2008) |
|             | zapA-R |                             |               |     |           |
| rsmA        | rsmA-F | TAGCGAGTGTG GACGAGTGGA AGCGAGGTGAA GAACGAGAA | 562 | 49 | Shi et al. (2016) |
|             | rsmA-R |                             |               |     |           |
| hpmA        | hpmA-F | ACGGCAAGGT CGTGCCCCAGTA ATGGCTAAT | 654 | 49 | Shi et al. (2016) |
|             | hpmA-R |                             |               |     |           |
| mrpA        | MRP-F  | ACACCTGCACCA TAGGAAAGATA CTGGTACA | 550 | 40 | Barbour et al. (2012) |
|             | MRP-R  | AAGTGATGAAG CTTAGTGATGG TGATGGGTG ATGAGAGTAAG TCACCC | | | |
| FliL        | FliL-F | CTCTGGCTCGTG GTGGGTGTCG GCGCTGCTC | 770 | 40 | Barbour et al. (2012) |
|             | FliL-R |                             |               |     |           |
| ucaA        | ucaA-F | GTAAAGTTGTT GCGCAAAC | 560 | 50 | Sosa et al. (2006) |
|             | ucaA-L |                             |               |     |           |
| pmfA        | pmfA-F | CAAATTAATCTA GAACCACCTG ATTAGAGGA TCCCTGAAAG | 618 | 54 | Zunino et al. (2003) |
|             | pmfA-R |                             |               |     |           |
| atfA        | atfA-F | CATATTGTCTTAG ACCTGCCCCATG CA | 382 | 50 | Zunino et al. (2000) |
|             | atfA-R |                             |               |     |           |

AT* anneling temperature

Table 2. Prevalence of P. mirabilis in collected diarrheal samples.

| Host | Number of samples | Number of positive samples (%) |
|------|------------------|-------------------------------|
| Dog  | 232              | 76 (32.76)                    |
| Mink | 216              | 62 (28.7)                     |
| Cattle | 86              | 20 (23.26)                    |
| Fowl | 80               | 18 (22.5)                     |
| Total| 614              | 176 (28.66)                   |

Table 3. Prevalence of ureC, zapA, rsmA, hpmA, mrpA, atfA, pmfA, FliL and ucaA genes in P. mirabilis.
Table 4. Antibiotic resistance pattern of the biofilm producing and non-producing *P. mirabilis*.

| Antibiotic | Biofilm producer (n = 162) | Non biofilm producer (n = 14) |
|------------|---------------------------|-----------------------------|
|            | resistanc e | intermediate | sensitive | resistanc e | intermediate | sensitive | P value |
| Doxycycline | 98(60.49%) | 78(48.15%) | 36(22.23%) | 14(100%) | 0(0%) | 0(0%) | 0.015 |
| Ampicillin | 92(56.79%) | 78(48.15%) | 36(22.23%) | 12(85.71%) | 2(14.29%) | 0(0%) | 0.078 |
| Ciprofloxacin | 90(55.56%) | 72(45.27%) | 36(22.23%) | 11(78.57%) | 0(0%) | 3(21.43%) | 0.084 |
| Streptomycin | 88(54.32%) | 72(45.27%) | 36(22.23%) | 10(71.42%) | 2(14.29%) | 2(14.29%) | 0.445 |
| Tetracycline | 85(52.47%) | 72(45.27%) | 36(22.23%) | 12(85.71%) | 2(14.29%) | 0(0%) | 0.34 |
| Piperacillin/ntazobactam | 84(51.85%) | 72(45.27%) | 36(22.23%) | 4(28.58%) | 2(14.29%) | 8(57.13%) | 0.15 |
| Cefotaxime | 78(48.15%) | 54(33.33%) | 30(18.52%) | 8(57.13%) | 6(42.87%) | 0(0%) | 0.255 |
| Nitrofurantoin | 67(41.36%) | 39(24.07%) | 36(22.23%) | 8(57.13%) | 2(14.29%) | 4(28.58%) | 0.492 |
| Sulfamethoxazole | 64(39.5%) | 36(22.23%) | 36(22.23%) | 12(85.71%) | 0(0%) | 2(14.29%) | 0.004 |
| Ceftriaxone | 62(38.27%) | 45(27.78%) | 55(33.95%) | 5(35.7%) | 3(21.43%) | 6(42.87%) | 0.776 |
| Polymyxin B | 61(37.66%) | 45(27.78%) | 55(33.95%) | 8(57.13%) | 2(14.29%) | 4(28.58%) | 0.357 |
| Cefazidime | 58(35.8%) | 42(25.93%) | 63(38.88%) | 4(28.58%) | 0(0%) | 10(71.42%) | 0.131 |
| Kanamycin | 57(35.19%) | 42(25.93%) | 63(38.88%) | 10(71.42%) | 4(28.58%) | 0(0%) | 0.005 |
| Gentamicin | 52(32.12%) | 42(25.93%) | 63(38.88%) | 8(57.13%) | 2(14.29%) | 4(28.58%) | 0.143 |
| Cefoperazone | 48(29.63%) | 45(27.78%) | 68(41.97%) | 8(57.13%) | 2(14.29%) | 4(28.58%) | 0.104 |
| Cephalotin | 45(27.78%) | 42(25.93%) | 80(49.38%) | 2(14.29%) | 0(0%) | 10(71.42%) | 0.03 |
| Meropenem | 42(25.93%) | 39(24.07%) | 90(55.55%) | 8(57.13%) | 0(0%) | 6(42.87%) | 0.061 |
| Levofloxacin | 39(24.07%) | 30(18.52%) | 93(57.41%) | 6(42.87%) | 0(0%) | 8(57.13%) | 0.117 |
| Imipenem | 36(22.58%) | 30(18.52%) | 102(62.9) | 0(0%) | 2(14.29%) | 12(85.71%) | 0.114 |

Table 5. Pathogenicity to mice in 32 of biofilm producing and non-producing *P. mirabilis* from animal with diarrhea.
### Figures

**Figure 1**

Antibiotic resistance phenotypes of *P. mirabilis* isolates examined in this study.

(A) Resistance rates of all isolates to 19 antibiotics. (B) Approximately 76.7% of the isolates exhibited multidrug or extensive drug resistance.