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Short Paper

Comparison of virulence genes in *Proteus* species isolated from human and pet turtle

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Summary

The current study was aimed to investigate the prevalence of *ureC*, *rsbA*, *zapA* and *mrpA* virulence genes using polymerase chain reaction (PCR) in *Proteus* spp. isolated from 5 commercially popular species of pet turtles and comparison of the *mrpA* gene sequences of *Proteus mirabilis* isolates with human clinical isolates. A total of 24 isolates in pet turtles were identified, comprised of *P. mirabilis* (15), *Proteus vulgaris* (7) and *Proteus hauseri* (2). The prevalence of *ureC*, *rsbA*, *zapA* and *mrpA* genes among all identified *Proteus* spp. isolates were 91.7%, 50%, 45.8% and 45.8%, respectively. The average percentage similarities of *mrpA* gene sequence of pet turtle *P. mirabilis* isolates to human urinary and respiratory isolates were 96.35% and 94.85%, respectively. The prevalence of virulence genes and high similarity of *mrpA* gene sequences between pet turtles and human *P. mirabilis* isolates revealed that though pet turtles are healthy, these animals may pose a potential risk of urinary and respiratory infections to humans.

Key words: *mrpA* gene, Pet turtles, *Proteus* spp., Virulence genes

Introduction

The genus *Proteus* is a Gram-negative bacillus that belongs to the Enterobacteriaceae family. Members of the genus *Proteus* are widespread in the environment and the gastrointestinal tract of human and animals (Hegazy, 2016).

Proteus is known as a nosocomial, opportunistic pathogen and is more common in community-acquired infections (Omoruyia and Evangelista, 2014). *Proteus mirabilis* and *P. vulgaris* have been reported to cause wound infections, respiratory tract infections and both community-acquired and catheter-associated urinary tract infections (UTI) (Li and Mobley, 2002; Trivedi *et al*., 2015). In addition, Hordijk *et al*. (2013) have reported urinary and kidney infections of companion animals, diarrhea in cats and dogs.

Virulence of the *Proteus* spp. is caused by several virulent factors and these virulent factors are regulated by virulent genes encoded in operons (Manos and Belas, 2006). This study focused on *zapA*, *rsbA*, *mrpA* and *ureC* genes. Since they have been identified to code most important virulent factors and have been found to be more common in previous studies (Abbas *et al*., 2015; Alsherees *et al*., 2016). One of the prominent features of *Proteus* spp. is the ability to swarm on solid surfaces. Even though several genes are associated with the swarming phenomenon as *cheW*, *gidA* and *cldA* genes, the *rsbA* gene is important for the swarming regulation (Rather, 2005).

Urease is the most important enzyme for kidney and bladder stone formation in *Proteus* infection. The *ureA*, *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, *ureG* and *ureR* genes on *ure* operon are responsible for the production process of urease enzyme and previous study pointed out *ureC* as a major gene, causative for urease production (Li and Mobley, 2002). The *zap* operon encoded by *zapA*, *zapB*, *zapC* and *zapD* genes is important for the production of protease, especially *zapA* for regulating *IgA* protease expression during the differentiation of swimmer cells to swarmer cells (Walker *et al*., 1999).

A variety of fimbriae have been detected in *P. mirabilis*. The vital type is MR/P fimbria encoded by *mrpA*, *mrpB*, *mrpC*, *mrpD*, *mrpE*, *mrpF*, *mrpG* and *mrpI* genes. The *mrpA* gene is significantly important to the pathogenicity, since it contributes numerous virulent factors such as adherence of bacteria to the epithelial tissue, biofilm formation, and swarming phenomenon (Rocha *et al*., 2007).

According to the comparison of genetic characteristics among bacterial isolates of human and animal origin, the issue of possible transmission risk to human has been discussed previously (Barbour *et al*., 2012). Nucleotide sequences of a few genes of human

isolated *P. mirabilis* were compared with chicken and dog isolates with regard to fimbriae (Barbour *et al*., 2012; Harada *et al*., 2014).

Nowadays, pet turtles are known as a potential risk in public health, and *Proteus* spp. cause anorexia, pneumonia, depression, and the death of pet turtles (Henriksen, 1972). Therefore, the objectives of this study were to investigate *mrpA*, *zapA*, *rsbA* and *ureC* genes in *Proteus* spp. isolated from pet turtles and to compare the acquired *mrpA* sequences of *P. mirabilis* with human clinical isolates.

Materials and Methods

Fifty-two pet turtles including 2 African side-neck turtles (*Pelusios castaneus*), 31 Chinese stripe-necked turtles (*Ocadia sinensis*), 10 river cooters (*Pseudemys concinna concinna*), 3 Western painted turtles (*Chrysemys picta belli*) and 6 yellow-bellied sliders (*Trachemys scripta scripta*) were purchased from pet shops and online markets. The turtles were under 4 weeks of age. The turtles were raised under laboratory conditions following the general husbandry method (Bluvias and Eckert, 2010).

Isolation and identification of *Proteus* spp. were performed according to standard procedure (Senior, 1997; Back *et al*., 2016). PCR amplification of *mrpA*, *zapA*, *rsbA* and *ureC* genes was performed using specific primers and PCR conditions given in Table 1.

Randomly selected five *mrpA* amplicons of *P. mirabilis* were purified using Expin™ PCR SV kit (GeneAll®, Korea) and sent to Cosmogenetech Co. Ltd., Daejeon, Korea for direct sequencing. The nucleotide sequences of the *mrpA* gene from turtles were compared with the sequences of the *mrpA* gene from human isolates (respiratory and urinary) previously reported by Barbour *et al*. (2012) and using BLAST option of NCBI (Basic Local Alignment Search Tool, BLAST v. 2.2.15, www.ncbi.nlm.nih.gov.)

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Results

Twenty-four isolates were positive in biochemical tests and 16S rRNA sequencing could identify them up to species level. Out of 24 bacterial isolates, 2, 7 and 15 isolates were identified as *P. hauseri*, *P. vulgaris* and *P. mirabilis*, respectively. The population of isolated bacteria predominantly consisted of *P. mirabilis* (Table 2).

The *UreC* was the most prevalent gene and was identified in twenty-two isolates (91.7%). However, the *ureC* gene was detected only in *P. mirabilis* and *P. vulgaris* with 100% detection rate in both species (Table

Table 1: Description of gene targets and the corresponding primers used for the specific virulent genes in PCR assay

Gene	Primer type	Primer sequence 5´-3´	Size bp	T (°C) [a]	References
mrpA	mrpAF	ACACCTGCCCATATGGAAGATACTGGTACA	550	40°C	Barbour *et al*. (2012)
	mrpAR	AAGTGATGAAGCTTAGTGATGGTGATGGTGATGAGAGTAAGTCACC			
rsbA	rsbAF	TTGAAGGACGCGATCAGACC	467	58°C	Abbas *et al*. (2015)
	rsbAR	ACTCTGCTGTCCTGTGGGTA			
ureC	ureCF	GTTATTCGTGATGGTATGGG	317	56.2°C	Ali and Yousif (2015)
	ureCR	ATAAAGGTGGTTACGCCAGA			
ZapA	ZapAF	ACCGCAGGAAAACATATAGCCC	540	59°C	Ali and Yousif (2015)
	ZapAR	GCGACTATCTTCCGCATAATCA			

[a] Annealing temperature

Table 2: Distribution of *Proteus* spp. isolated from pet turtles

Gene	Number (%) of positive strains for genes			Total positive strains (n=24)
	P. mirabilis (n=15)	*P. vulgaris* (n=7)	*P. hauseri* (n=2)	
ureC	15 (100)	7 (100)	0 (0)	22 (91.7)
rsbA	12 (80)	0 (0)	0 (0)	12 (50)
zapA	11 (73.3)	0 (0)	0 (0)	11 (45.8)
mrpA	11 (73.3)	0 (0)	0 (0)	11 (45.8)

Table 3: Distribution of virulent genes of *Proteus* spp. isolated from pet turtles

Turtle	Number (%) of isolates			Isolation rate of *Proteus* spp. (%)
	P. mirabilis	*P. vulgaris*	*P. hauseri*	
Chinese stripe-necked turtle (n=31)	7 (22.6)	4 (12.9)	0 (0)	11 (35.5)
River cooter (n=10)	6 (60)	2 (20)	1 (10)	9 (90)
Yellow-bellied slider (n=6)	1 (16.7)	0 (0)	1 (16.7)	2 (33.4)
Western painted turtle (n=3)	1 (33.3)	0 (0)	0 (0)	1 (33.3)
African side-neck turtle (n=2)	0 (0)	1 (50)	0 (0)	1 (50)
Total (n=52)	15 (28.8)	7 (13.5)	2 (3.8)	24 (46.1)

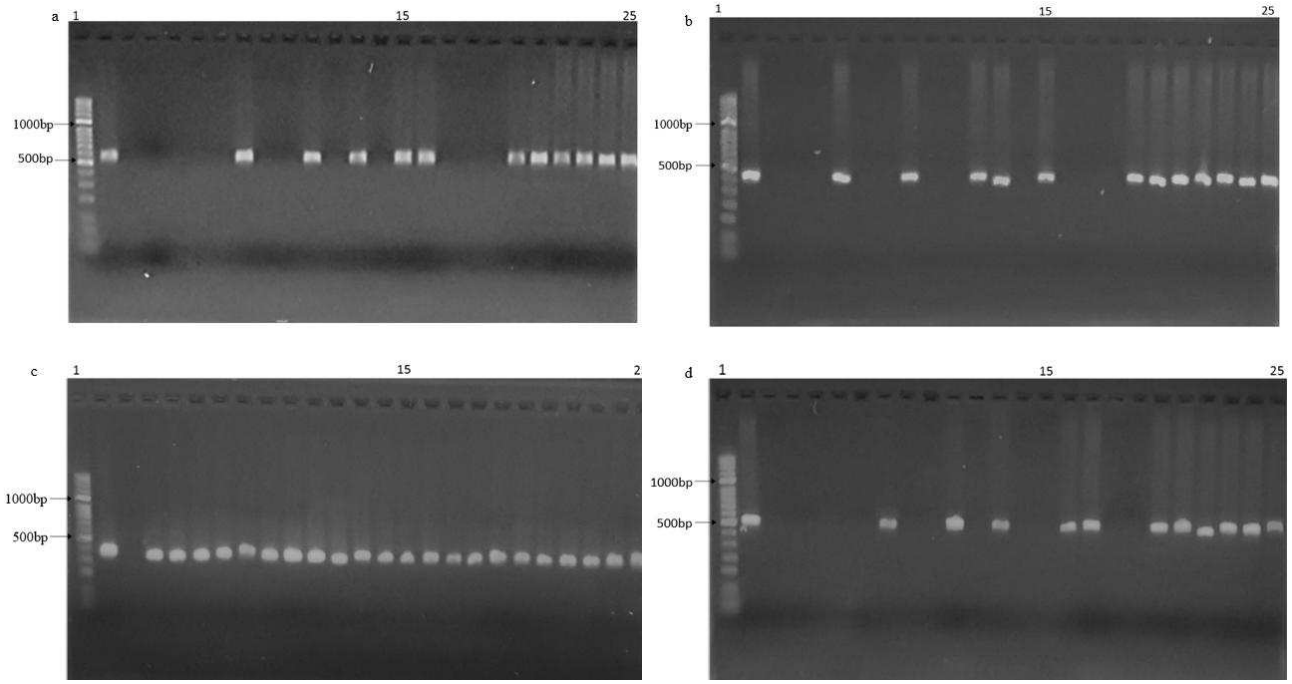

Fig. 1: Amplicons detection by PCR in *P. mirabilis* and *P. vulgaris* of pet turtle isolates (Lanes 4-25). **a**: *mrpA* gene, **b**: *rsbA* gene, **c**: *ureC* gene, and **d**: *zapA* gene. Other lanes are for the negative control (Lane 3), *P. mirabilis* reference isolate (American Type Culture Collection) (Lane 2), and genetic base-pair ladder (Lane 1)

Table 4: Average similarity percentages of *mrpA* sequences of *P. mirabilis* recovered from pet turtles in comparison to human respiratory and urinary clinical isolates

Turtle isolates	Mean % similarity in *mrpA* amplicon sequences of pet turtles to human isolates									
	Human urinary isolates				Mean	Human respiratory isolates				Mean
	1	2	3	4		1	2	3	4	
1	98.0	98.0	98.0	98.0	98.0	96.0	98.0	96.0	98.0	97.0
2	95.0	95.0	95.0	95.0	95.0	93.0	95.0	94.0	95.0	94.3
3	97.0	96.0	98.0	96.0	96.8	96.0	96.0	95.0	94.0	95.3
4	98.0	98.0	96.0	95.0	96.8	93.0	95.0	93.0	95.0	94.0
5	93.0	95.0	98.0	95.0	95.3	94.0	93.0	95.0	93.0	93.8
Mean	96.2	96.4	97.0	95.8		94.4	95.4	94.6	95.0	
Mean of means					96.4*					94.9**

*, ** Mean of means in a row followed by different symbols are significantly different (P<0.05)

3). Among four virulent genes, *rsbA* was detected at the ratio of 50% (12/24) while *zapA* and *mrpA* exhibited similar percentage, 45.8% (11/24). 80% of *rsbA*, 73.3% of *zapA*, and 73.3% of *mrpA* were observed in *P. mirabilis* (Fig. 1). In *P. vulgaris ureC* was the only gene detected and none of the genes were identified in *P. hauseri* isolates.

Mean similarity percentages with regard to *mrpA* gene of pet turtles and four human urinary *P. mirabilis* isolates were 96.2%, 96.4%, 97.0%, and 95.8%, respectively. Besides, human respiratory and turtle isolates showed 94.4%, 95.4%, 94.6%, and 95.0% of similarity percentages. Mean of means was calculated using similarity percentages and it was 96.4% in urinary isolates and 94.9% in respiratory isolates (Table 4).

Discussion

Proteus spp. was detected in many wild and domestic animals particularly with no clinical signs (Drzewiecka, 2016). Since *Proteus* spp. is an opportunistic pathogen, even healthy turtles can be asymptomatic carriers of the pathogen (Hossain *et al*., 2016). In the present study, *P. mirabilis* was the predominant species followed by *P. vulgaris* as 62.5% (15/24) of *P. mirabilis*, 29.17% (7/24) of *P. vulgaris* and 8.34% (2/24) of *P. hauseri*. Dalia (2015) has reported the same results with clinical isolates.

The current study was mainly focused on the detection of virulence genes. Several virulence determinants contribute to the pathogenicity of *Proteus* spp. (Schaffer and Pearson, 2015). The *ureC* gene is responsible for the elevation of pH of urine and resulting in stone formation (Mohammed *et al*., 2014). In this study, *ureC* amplification of *P. mirabilis* and *P. vulgaris* was 100%, which reveals a higher frequency of *ureC* compared with the rest of the genes. It indicates the crucial role of *ureC* for the virulence of the *Proteus* spp.

Regarding *ureC* gene, these results are in agreement with a previous study, indicating the presence of *ureC* approximately 96.6% among isolates obtained from patients with UTI (Ali and Yousif, 2015).

The *rsbA* gene could not be amplified in *P. vulgaris* and *P. hauseri*. However, the majority of positive strains were *P. mirabilis* and it is in line with the previous studies (Rather, 2005; Abbas *et al*., 2015). In the current study, a phenotypic character of swarming, the bull's-eye ring was exhibited by all the isolates. Nevertheless, swarming regulated genes are not necessarily required for swarming, for the reason that so many genes and operons are involved in the process (Pearson *et al*., 2010).

The *zapA* gene which is coding for protease enzyme production and *mrpA* gene which is mainly important for fimbriae gene were identified in similar number of isolates 11 (45.8%). The *zapA* and *mrpA* could not be detected in *P. vulgaris* and *P. hauseri* isolates. Nevertheless, the frequency of both genes in *P. mirabilis* was 73.3% in disagreement with previous studies which were 30% (Alsherees *et al*., 2016) and 100% (Ali and Yousif, 2015) in clinical *P. mirabilis* isolates. Although the prevalence of *mrpA* gene in previous studies was small in percentage values, the most important gene among tested gene is *mrpA* due to its contribution to several virulent factors (Abbas *et al*., 2015). Therefore, *mrpA* was the selected gene for comparison of gene sequences.

Furthermore, comparison of *mrpA* amplicon sequences of pet turtles to human isolates revealed similarity percentages. Fimbriae are imperative for several virulent factors of *Proteus* species. Among 6 fimbriae types and 17 fimbrial operons, MR/Pis the most important fimbria (Różalski *et al*., 2012). This type of fimbria is encoded by *mrp* operon containing 10 genes located on the bacterial chromosome. The main structural subunit of these fimbriae is the MrpA protein. Though other fimbriae are present only in *P. mirabilis*, the *mrp* operon was shown not only in *P. mirabilis* strains but also in other species of the *Proteus* genus. In our results, comparison of pet turtles to human urinary isolates, using *mrpA* gene of *P. mirabilis* represents 96.40% of mean similarity and pet turtles to human respiratory isolates using the same gene of *P. mirabilis* represents 94.90% of mean similarity.

Previous studies proved that direct contact even with healthy turtles can transmit diseases to humans and if people, especially immune suppressed adults and children, have direct physical contact with turtles and aquarium water, there might be a potential risk to transmit pathogens and to cause urinary infections (Mermin *et al*., 2004; Hidalgo-Vila *et al*., 2007).

In conclusion, the high prevalence of *Proteus* species, especially *P. mirabilis* in pet turtles, and their similarity of virulence gene characteristics with human clinical isolates could be emphasized. Thus the potential health risk of infection of *Proteus* spp. from pet turtles should not be underestimated. Expanding this research further with more virulent genes could be important for a better understanding of the pathogenicity of *Proteus* spp. harbored by pet turtles. According to our knowledge, this is the first report designed to detect *zapA*, *rsbA*, *mrpA* and *ureC* genes in *Proteus* isolates of pet turtles.

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Conflict of interest

The authors declare no conflict of interests.

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