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Research Article

Molecular Characteristics of *Pasteurella multocida* Strains Isolated from Poultry in China and Genetic Analysis of Strains in Terms of the *tonB* Gene

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Abstract

Pasteurella multocida (P. multocida) causes fowl cholera in birds and the pathogenesis and virulence factors involved are still poorly understood. TonB protein is a periplasmic protein prevalent in a large number of Gram-negative bacteria, as in Pasteurella, which is believed to be responsible for the import of ferric iron complexes across the outer membrane and plays a role as an important virulence factor to help bacteria to obtain nutrients. In this study, a total of 23 isolates of *P. multocida* were obtained from 172 cases of clinical respiratory disease in duck and geese, giving an isolation rate of 13.4%. *P. multocida* Serogroup A was isolated from 22 cases (95.7%), whereas serogroup F was from 1 case (4.3%). All isolates were analyzed for their susceptibility to 15 antibiotics and the presence of 18 genes for virulence factors. The susceptibility profiles suggested that Tetracycline, Cephalosporin, Enrofloxacin, and Aminoglycosides were the drugs most likely to be active against *P. multocida*. However, 78.3% and 52.2% of poultry strains were resistant to Penicillin and Sulfisoxazole, respectively. PCR results showed that *ptfA, fimA, tonB, fur, hgbA, hgbB, sodA, sodC, pmHAS, nanH, nanB, plpB* and *ompH* genes occur in most poultry strains of *P. multocida*. The virulence genes such as *toxA, tadD, hsf-1, pfhA* and *ompA* were each present in <73.9% of strains. The *tonB* gene was detected in all 23 clinical strains of *P. multocida* which were recovered from different hosts and harboring different serotypes. Phylogenetic analysis indicated that these *tonB* genes of *P. multocida* from different hosts and harboring different serotypes have close genetic relationship with a high similarity. Multiple sequence alignment demonstrated that TonB protein of Gram-negative bacteria exists in multiple conserved sites, such as -xSSGx-, -YP-, -LD- and xA[A/V]Lx motif. These findings provide clinical data into the epidemiological and molecular characteristics of avian *P. multocida* isolates and provide a reference for the researches of drug target in *P. multocida*.

Keywords: *Antimicrobial susceptibility, Capsular antigens, Pasteurella multocida, tonB gene, Virulence gene, Cloning, Phylogenetic analysis*

Çin'de Kanatlı Hayvanlardan İzole Edilen *Pasteurella multocida* Sışlarının Moleküler Karakterizasyonu ve *tonB* Geni Açısından Genetik Analizi

Özet

Pasteurella multocida (P. multocida) kuşlarda kanatlı kolerasına neden olur ve hastalıkta virulans faktörleri hala tam olarak anlaşılamamıştır. TonB proteini *Pasteurella* da dahil pek çok Gram-negatif bakteride yaygın olarak bulunan periplazmik bir protein olup membran dışına ferrik demir komplekslerini taşımakta görevlidir ve bakterilerde besin sağlamaya yardım etmek suretiyle önemli bir virulans faktörü olarak rol oynar. Bu çalışmada, 172 klinik solunum hastalıklı ördek ve kazdan toplam 23 *P. multocida* izolatı elde edilerek %13.4'lük izolasyon oranı sağlandı. *P. multocida* Serogrup A 22 vakadan izole edilirken (%95.7), serogrup F 1 vakadan izole edildi (%4.3). Tüm izolatlar 15 antibiyotiğe karşı duyarlılıklarına ve virulans faktör olarak 18 genin mevcudiyetine göre analiz edildi. Duyarlılık profili, *P. multocida*'nın tetrasiklin, sefalosporin, enrofloksasin ve aminoglikozidlere karşı duyarlı olduğunu gösterdi. Ancak, elde edilen suşların %78.3'ü penisiline ve %52.2'si sulfisoksazola karşı dayanaklıydı. PCR sonuçları çoğu kanatlı *P. multocida* suşlarında *ptfA, fimA, tonB, fur, hgbA, hgbB, sodA, sodC, pmHAS, nanH, nanB, plpB* ve *ompH* genlerinin olduğunu gösterdi. *toxA, tadD, hsf-1, pfhA* ve *ompA* gibi virulans genlerinin her biri suşların <73.9%'da mevcuttu. *tonB* geni *P. multocida*'nın tüm 23 klinik suşlarında farklı konakçılardan elde edildi ve farklı serotipleri içermekteydi. Filogenetik analiz sonucunda farklı konakçılardan elde edilen ve farklı serotipleri içeren *P. multocida tonB* genlerinin yakın genetik akrabalıkları olduğunu ve yüksek oranda benzerlik gösterdiği belirlendi. Multiple sekans hizalama ile Gram-negatif bakterilerin TonB proteinin -xSSGx-, -YP-, -LD- ve xA[A/V]Lx motif gibi çok sayıda korunmuş bölgesinin olduğu gösterildi. Elde edilen bulgular avian *P. multocida*'nın epidemiyolojik ve moleküler karakterizasyonu için klinik veri ile *P. multocida*'ya karşı ilaç üretiminde araştırmacılar için bir referans sağlamıştır.

Anahtar sözcükler: *Antimikrobiyal duyarlılık, Kapsül antijenleri, Pasteurella multocida, tonB geni, Virulans geni, klonlama, Filogenetik analiz*

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INTRODUCTION

Pasteurella multocida (P. multocida) is a Gram-negative pathogen causing severe zoonosis and it is widely believed that this bacterium through tissues of the respiratory and digestive tracts results in the occurrence of pasteurellosis [1,2]. *P. multocida* is associated with the several distinct diseases. These can be classified into the two general groups including hemorrhagic septicemia and respiratory system diseases, for instance, avian cholera, hemorrhagic septicemia in ungulates, atrophic rhinitis in pigs and snuffles in rabbits [3]. *P. multocida* strains are classified into the serogroups (A, B, D, E and F) based on the capsule antigens, and there is a significant association between various capsular serotypes and pathogenicity [4]. The pathogenicity of *P. multocida* is associated with the different virulence factors. A number of virulence factors identified of *P. multocida* to date include fimbriae, adherence and colonization factors *(ptfA, fimA, hsf-1, pfhA* and *tadD)*, iron-regulated and acquisition proteins *(tonB, hgbA, hgbB* and *fur)*, extracellular enzymes such as neuraminidase *(nanB* and *nanH)*, hyaluronidase *(pmHAS)* and superoxide dismutase *(sodA* and *sodC)*, dermonecrotoxin *(toxA)* and a variety of Outer Membrane Proteins (OMPs) such as protectins *(ompA, ompH* and *plpB)* [5-10]. These pathogenic factors help *P. multocida* to invade hosts and damage tissues.

Iron (Fe^{2+}), is an essential nutrient for both pathogenic microorganisms and their hosts [11]. It is a cofactor for a large number of important enzymes, involved in many fundamental cellular processes, including electron transfer, cell respiration, and superoxide metabolism [8]. In addition, it is a pivotal component of the innate immune response through its role in the generation of toxic oxygen and nitrogen intermediates [12]. Pathogens are involving growth, proliferation and disease processes, can not directly use Fe^{2+} from the host body, but compete with the host for Fe^{2+} by iron uptake-related proteins to fulfill their iron needs [13]. TonB protein, a periplasmic protein, plays an important role in energy-dependent transport of iron siderophores of Gram-negative bacteria as well as a virulence factor [14]. Many studies have indicated that the Gram-negative bacteria can synthesize siderophores chelate iron with high affinity in cytoplasm. This compound is chelated with Fe^{2+} of iron-binding proteins in hosts. Then the TonB protein, with the help of the ExbB/ExbD complex, transduces the energy of the cytoplasmic membrane proton-motive force (pmf), activates the siderophore receptor to allow substrate internalization into the periplasmic space and enters to cytoplasm through the specific ATP binding cassette (ABC) transporter ulterior. Consequently the pathogen is able to reunite the iron source [8,15,16]. Many other pathogenic bacteria can also directly recognize heme or heme binding proteins by outer membrane receptors for heme, and transfer the host's heme to the cytoplasm depend on the ExbB-ExbD-TonB system for energy as well as the specific ABC transporters, in this way, they obtain the iron source [17,18]. Therefore, some studies have focused on the *Haemophilus influenzae* TonB protein and have been demonstrated that TonB protein plays a crucial role in utilization of heme and produces invasive disease of *H. influenzae* [19].

In this report, we aimed to identify the capsular serotypes of *P. multocida* from duck and geese in Sichuan-Chongqing regions, China. Moreover, the analysis of genetic characteristics of TonB protein of *P. multocida* was displayed.

MATERIAL and METHODS

Samples

During the period from 15th February to 3rd December 2016, a total of 172 liver samples were collected under aseptic conditions from ducks (n = 121) with 1 to 2 months age and geese (n = 51) with 2 to 3 months age suffering from respiratory manifestations, such as nasal discharge, cough and dyspnea. The samples were collected from different farms located in Chongqing and Sichuan, China, and transferred under complete aseptic condition to the College of Science Laboratory at Rongchang Campus of Southwest University for standard bacteriological examination and molecular detection of *P. multocida*.

Bacterial Isolation and Identification

Each liver sample was plated on Luria-Bertani (LB, Oxoid, Thermo Fisher Scientific, China) agar supplemented with 5% defibrinated sheep blood. All plates were incubated at 37°C under appropriate air conditions (aerobic conditions and 90% relative humidity) for 24 h. Afterward, the isolates were purified and cultured by standard methods for rapid primary identification of *P. multocida* by PCR using the specific primers Kmt1 stated in Townsend KM et al.[20].

Capsular Types and Virulence Genes of P. multocida

The capsular types of *P. multocida* were confirmed by multiplex PCR with capsule-specific primer pairs (A and F) [21]. All isolates were analyzed with PCR in terms of the 18 virulence-associated genes which were reported specifically to *P. multocida* previously [22-24]. The primers [23] used in the virulence factors assay for *P. multocida* were synthesized commercially with finishing done by BaiLiGo Biotechnology Shanghai Co., Ltd.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *P. multocida* isolates was determined using the Kirby–Bauer disc diffusion method [25] after strains were cultured on Mueller-Hinton agar (Oxoid, Thermo Fisher Scientific, China). Amikacin (10 μg), Ampicillin (30 μg), Cefalexin (30 μg), Cefradine (30 μg), Enrofloxacin (5 μg), Gentamicin (10 μg), Kanamycin (30 μg), Amoxicillin (10 μg), Doxycycline (30 μg), Neomycin (30 μg), Erythromycin

(15 µg), Carbenicillin (100 µg), Penicillin (10 µg), Tetracycline (30 µg), Sulfisoxazole (300 µg) are the antimicrobial agents (Oxoid) which were provided from Thermo Fisher Scientific CO., LTD. As defined as the multidrug-resistance (MDR) is an acquired non-susceptibility to at least one agent in three or more antimicrobial categories [26].

Cloning of tonB Gene

DNAs of 23 *P.multacida* strains were extracted by a commercially available kit (TaKaRa Biotechnology Dalian Co. Ltd.). On the basis of complete genome sequences (NC002663.1) of *P. multocida* registered in GenBank, we designed the primer pairs (tonB-F: CCGGAATTCATGATA GATAAAAGTCGT; tonB-R: CCCAAGCTTTTAATTTGTGATT-CTGAA) to amplify the *tonB* gene, and the yielded amplified product was expected about 771bp. Each PCR contained a total volume of 50 µL, including 25 µL Premix Ex Taq polymerase (TaKaRa Biotechnology Dalian Co., Ltd), 8 µL DNA template, 2 µL of each primer (20 µmol/L) and 13 µL ddH_2O. The PCR thermal condition included 10 min initial denaturation step at 94°C, followed by 30 cycles of the following steps as 30 sec denaturation at 94°C, 45 sec annealing at 53°C, 1 min of extension at 72°C and a final 5 min extension step at 72°C. The amplicons were cloned and sequenced subsequently. PCR reaction products were ligated into the pMD-19 T vector (Takara, Dalian, China), transformed into DH5α cells, and plated onto Luria-Bertani (LB, Oxoid, Thermo Fisher Scientific, China) agar plates containing Ampicillin (100 µg/mL). For each *P. multocida* isolate, three independent colonies derived from two independent PCR reactions were sequenced to obtain a consensus sequence.

Phylogenetic Analysis of tonB Gene in P. multocida

To investigate the evolutionary relationship of *tonB* gene in *P. multocida*, phylogenetic analysis was performed by the Neighbour-Joining (NJ) and Maximum-Likelihood (ML) methods implemented in MEGA7.0 [27]. Conserved regions were determined using the Gblocks program [28]. The best-fitting nucleotide substitution model with the Akaike information criterion (AIC) score was determined using jModelTest 2.1.7 [29]. NJ and ML analyses of *P. multocida tonB* gene from different hosts were performed under the Hasegawa-Kishino-Yano + Has Invariant sites (HKY+I) model, and the robustness of the tree topology was assessed with 1,000 bootstrap replicates. NJ and ML analyses of *P. multocida tonB* gene from different sero-groups were performed under the Hasegawa-Kishino-Yano (HKY) model, and the robustness of the tree topology was assessed with 1,000 bootstrap replicates.

Phylogenetic Analysis of TonB Protein in Bacteria

We downloaded 3000 bacteria genomes from the Entrez Genome Project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), from these we sorted for 1330977 protein sequences and built a local protein database. Using cloned 23 TonB protein sequences of *P. multocida* in our study to construct a TonB Hidden Markov Model (TonB-HMM) by performing hmmbuild program of HMMER 3.0 software [30]. Retrieving the local protein database based on TonB-HMM, the screening criteria is e-value $<10^{-5}$. The screened protein sequences are considered to be predicted TonB protein sequences. All retrieved sequences were subjected to the multiple sequence alignment using L-INS-i method implemented in MAFFT7.1 software [31], and then using ML method with 500 bootstrap replicates to constructed phylogenetic tree implemented in MEGA7.0 [27].

Functional Effect of Point Mutations

To study the functional effect of point mutations on TonB protein sequence, one chosen representative TonB protein sequence was analyzed using PredictProtein (http://ppopen.rostl-ab.org/) based on the SNAP2 method, and the accuracy of results about functional effect of point mutations can be reached 82% [32].

RESULTS

Prevalence of P. multocida in Duck and Goose Clinical Samples

In this study, *P. multocida* was isolated from 23 (13.4%) out of 172 liver samples collected from duck and geese with clinical respiratory disease. Molecular identification of the recovered strains with the specific primer (Kmt1) revealed positive amplification of 457 bp fragments for all isolates *(Fig. 1)*. As the results of multiplex PCR specific for capsular antigens of *P. multocida,* 22 strains (95.7%) were found type A with a 1044 bp amplified fragment, whereas 1 strain (4.3%) was type F with 851 bp length fragment.

Antimicrobial Susceptibility

The antimicrobial susceptibility results of *P. multocida* isolates are described in *Table 1*. There was no drug which can reach to 100% efficiency in inhibition of the bacterial growth, but the isolates showed sensitivity to 50% of the antimicrobials, except sulfisoxazole and penicillin. 43.5% of the *P. multocida* isolates were found resistant to three or more drugs in different categories in this study.

Distribution of Virulence Genes

Among the 23 *P. multocida* isolates, the 18 virulence gene regions ranged in prevalence as 0% *(toxA)* to 100% *(tonB) (Table 2)*. Iron acquisition factors *(tonB*, *fur*, *hgbA* and *hgbB)*, adhesins-encoding *(ptfA* and *fimA)*, superoxide dismutases *(sodA* and *sodC)*, porin and outer membrane proteins *(plpB* and *ompH)*, neuraminidase *(nanB* and *nanH)* and hyaluronan synthase *(pmHAS)* genes were each found in more than 82.6% of the isolates. This shows that these virulence genes are highly prevalent among isolates of *P. multocida* recovered from poultry. In contrast, 5 genes *(toxA*, *tadD*, *hsf-1*, *pfhA* and *ompA)* were detected as 0% to

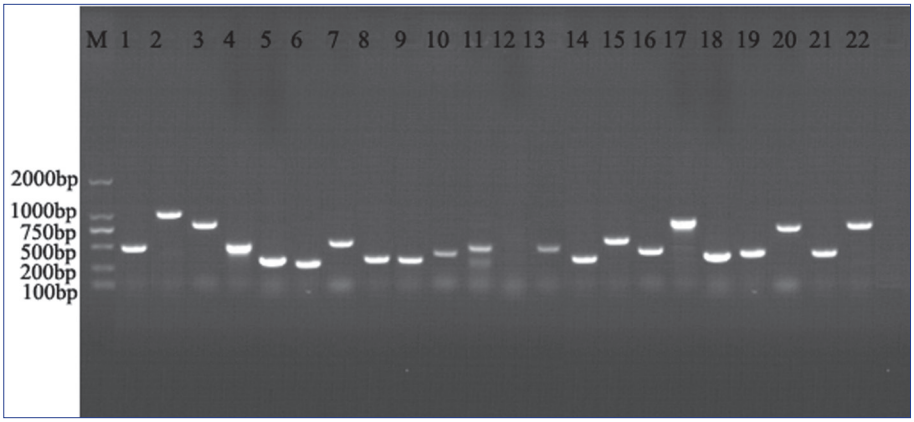

Fig 1. PCR identification, serological typing and the 18 virulence-associated genes analysis of *P. multocida* strains isolated from poultry. M: DNA Marker DL2000 (Takara, Dalian, China); 1:PCR identification for *P. multacida* (KMT gene); 2: PCR identification for capsular type A; 3: PCR identification for capsular type F; 4-21: PCR products of *ompH*, *plpB*, *ompA*, *nanB*, *nanH*, *sodC*, *sodA*, *pmHAS*, *toxA*, *ptfA*, *pfhA*, *hsf-1*, *tadD*, *fimA*, *fur*, *hgbA*, *hgbB* and *tonB* gene, respectively; 22: The complete fragment of *tonB* gene of *P. multocida*

Table 1. *The antimicrobial susceptibility patterns of P. multocida strains from poultry*

Antimicrobial	Susceptible	Non-Susceptible
Gentamicin	82.6% (19/23)	17.4% (4/23)
Kanamycin	91.3% (21/23)	8.7% (2/23)
Amoxicillin	56.5% (13/23)	43.5% (10/23)
Doxycycline	82.6% (19/23)	17.4% (4/23)
Neomycin	91.3% (21/23)	8.7% (2/23)
Erythromycin	52.2% (12/23)	47.8% (11/23)
Cefalexin	91.3% (21/23)	8.7% (2/23)
Cefradine	95.7% (22/23)	4.3% (1/23)
Sulfisoxazole	47.8% (11/23)	52.2% (12/23)
Carbenicillin	65.2% (15/23)	34.8% (8/23)
Penicillin	21.7% (5/23)	78.3% (18/23)
Amikacin	87.0% (20/23)	13.0% (3/23)
Ampicillin	56.5% (13/23)	43.5% (10/23)
Tetracycline	95.7% (22/23)	4.3% (1/23)
Enrofloxacin	91.3% (21/23)	8.7% (2/23)

Table 2. *Distribution of virulence factors of 23 strains P. multocida*

Virulence-Associated Genes	Detection (n=23)	Not Detected (n=23)
ompH	21 (91.3%)	2 (8.7%)
ompA	17 (73.9%)	6 (26.1%)
plpB	22 (95.7%)	1 (4.3%)
nanH	19 (82.6%)	4 (17.4%)
nanB	19 (82.6%)	4 (17.4%)
sodA	21 (91.3%)	2 (8.7%)
sodC	21 (91.3%)	2 (8.7%)
pmHAS	19 (82.6%)	4 (17.4%)
toxA	0 (0.0%)	23 (100%)
ptfA	19 (82.6%)	4 (17.4%)
pfhA	15 (65.2%)	8 (34.8%)
tadD	8 (34.8%)	15 (65.2%)
hsf-1	13 (56.5%)	10 (43.5%)
fimA	22 (95.7%)	1 (4.3%)
fur	22 (95.7%)	1 (4.3%)
hgbA	21 (91.3%)	2 (8.7%)
hgbB	20 (87.0%)	3 (13.0%)
tonB	23 (100%)	0 (0.0%)

73.9% percentage, and the *toxA* gene was not detected in any of the 23 studied clinical strains.

Cloning of tonB Gene

The amplified PCR product of 23 *P. multocida* isolates was 771bp as expected *(Fig. 1)*. Then the amplified product was cloned and sequenced. The 23 sequences were deposited in the GenBank database under accession numbers KY748240 - KY748250, KY623668- KY623679.

Alignment and Phylogenetic Analysis of tonB Gene in P. multocida

Phylogenetic trees with similar topologies were obtained by the two aforementioned methods (NJ and ML). As shown in *Fig. 2A*, the 22 *tonB* gene sequences of *P. multocida* deriving from different hosts fell into four distinct groups, called as Group I, Group II, Group III and Group IV. Eight isolates of this study (KY623677, KY623668, KY623670, KY623671, KY623676, KY623675, KY623669 and KY623674), poultry strain (LUCZ01000003) and *Capra aegagrus hircus* strain (AFRS01000078) belonged to Group I, whereas ovine strain (ARNZ01000046) and *Oryctolagus cuniculus* strain (NZ_MTIH01000003, NZ_MTIL01000003 and NZ_MTIF 01000003) belonged to Group II. Alpaca strain (KP660851), pig strain (CP001409), bovine strain (ARWR01000002) belonged to Group III and pig strain (CP003328) and bovine strains (ALBZ01000087, AROA01000190 and NZ_ JQEB01000010) belonged to Group IV. One rare strain from human (NBTJ01000001) did not belong to any group. As shown in *Fig. 2B*, the 22 *tonB* gene sequences of *P.multocida* deriving from different serogroups fell into two distinct groups, called as Group I and Group II. Serogroup B belonged Group II and the rest of serogroups clustered in Group I.

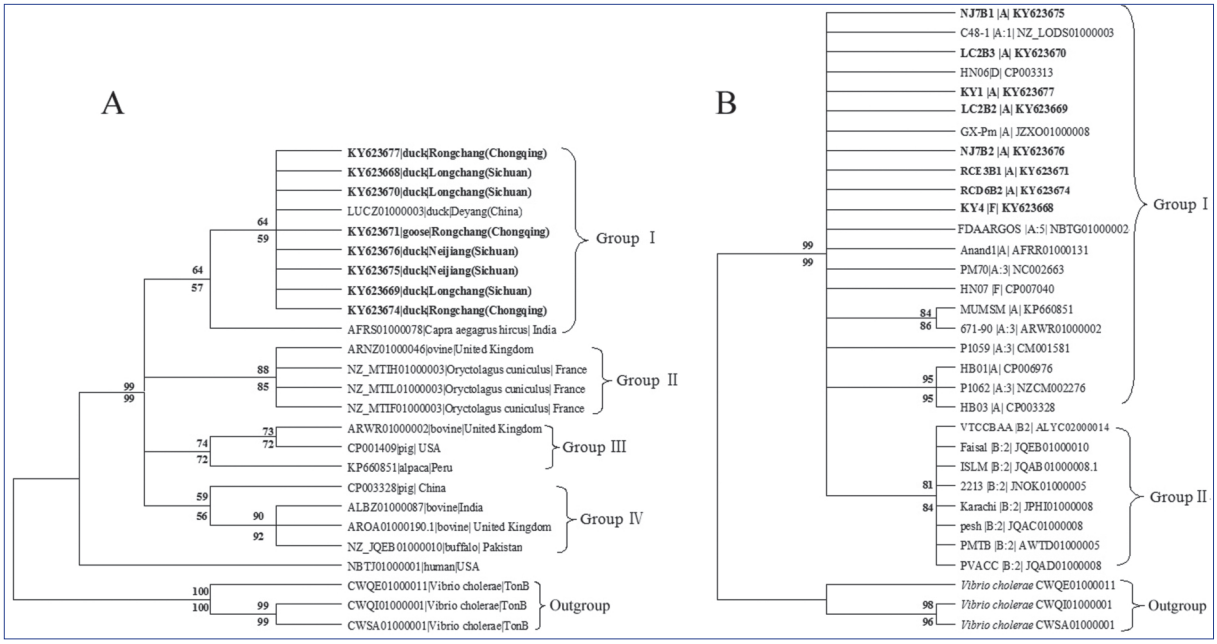

Fig 2. Evolutionary relationship of the *P. multocida tonB* gene from different hosts and different serotypes.
The NJ and ML bootstrap percentages are given above and below branches. The 8 *P. multocida* isolates from this study were indicated in bold font. The 3 *Vibrio cholerae* isolates, NC_003623, NC_004440 and NC_003839, were used as outgroups; (A) Phylogenetic tree shows genetic relationships among the *P. multocida tonB* gene from different hosts; (B) Phylogenetic tree shows genetic relationships among the *P. multocida tonB* gene from different serotypes

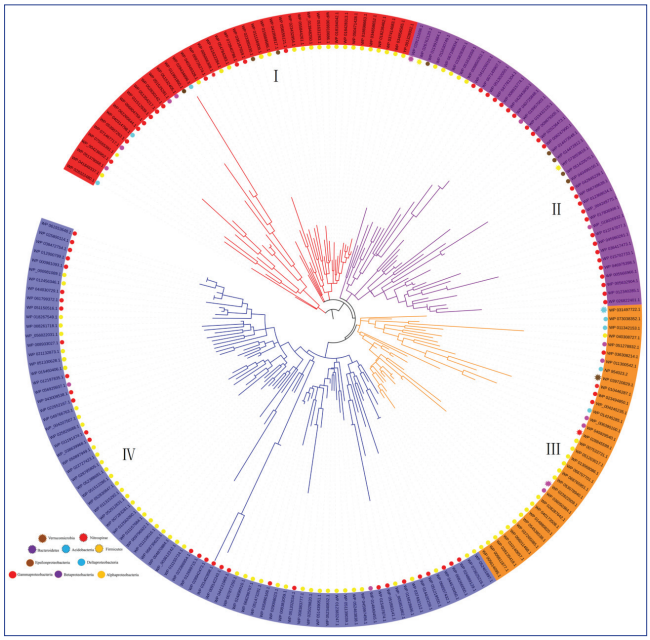

Fig 3. Phylogenetic tree of 196 typically bacterial TonB protein sequences. The numbers represent GenBank accession numbers. Different colors in the tree represent different groups of TonB: Red represents Group I, purple represents Group II, orange represents Group III, blue represents Group IV. The trees are based on the MAFFT-derived multiple sequence alignments (MSA). The tree was drawn with the iTOL program

Phylogenetic Analysis of TonB Protein in Bacteria

We obtained 302 significant sequences (E-value $<10^{-5}$) after retrieving the local protein database based on the TonB-HMM model, after that, 196 sequences were TonB protein sequences through screening and correcting. According to the phylogenetic tree *(Fig. 3)*, 196 sequences derived from 6 phyla, which contains *Proteobacteria* (187 sequences) and 9 sequences distributed among *Firmicutes, Nitrospirae, Acidobacteria, Bacteroidetes* and *Verrucomicrobia*. In *Proteobacteria*, 88 sequences were derived from *Alphaproteobacteria*, 71 sequences from *Gammaproteobacteria*, 13 sequences from *Betaproteobacteria*, 8 sequences from *Epsilonproteobacteria* and 7 sequences from *Deltaproteobacteria*. The proportions of them were 47.06%, 37.97%, 6.95%, 4.28% and 3.74%, respectively. So we knew that the bacteria shared a high similar sequence with the *P. multocida* TonB protein sequence were mainly concentrated in *Alphaproteobacteria* class of *Proteobacteria* phylum.

Multiple Sequence Alignment and Point Mutation Analysis

The phylogenetic tree was constructed with 4 lineages, as shown in *Fig. 4A*. According to the *Fig. 4A*, the -xSSGx- and -YP- motifs were the best conserved portion in these 4 lineages although there was a great variation of TonB protein sequences among different species. The -LD- motif existed in cluster 1, 2 and 4, while the -xA[A/V]Lx- motif distributed in cluster 1, 2 and 3, and these motifs were relatively conserved. The protein sequence of representative cloned strain (KY748245) was analyzed for the point mutation. The results showed that 3 amino acids of -xSSGx- motif in this cloned strain were highly conserved, and it is known if they are replaced by any

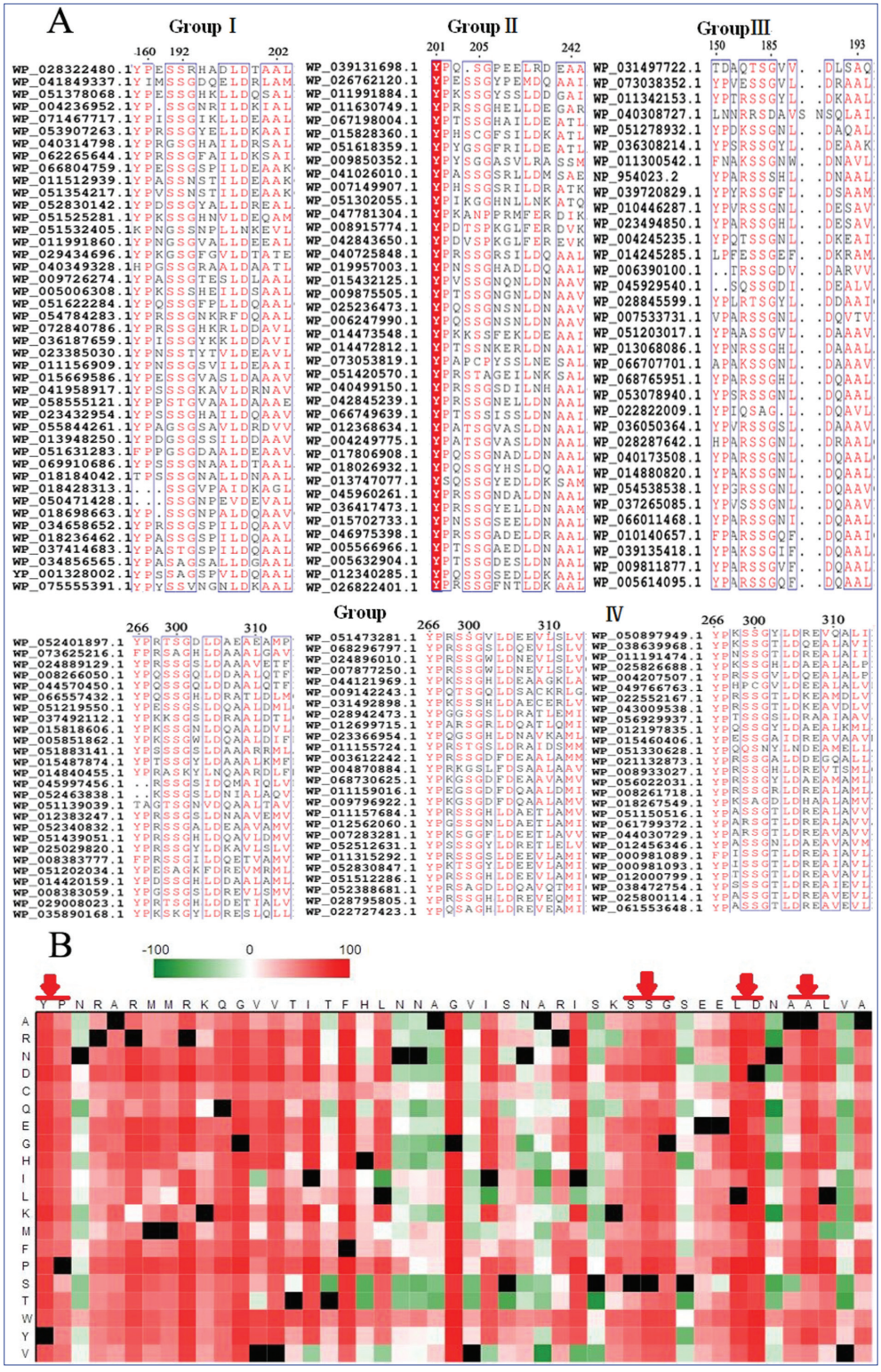

Fig 4. Alignment of amino acid sequences of bacterial TonB in each group and analysis of the effect of point mutations of KY748245. A: The -xSSGx- and -YP- motifs existed in four groups, the -LD-motif existed in Group I, II, IV, and the -xA[A/V]Lx-motif existed in Group I, II , III; B): Deep red (>50): Have great influence on function if replaced; Green and White (<50): Have relatively small influence on function if replaced; Black: Wild type

amino acid, there must be some serious impacts on the function of TonB protein in bacteria *(Fig. 4B)*. In addition, the amino acids in -LD- and -xA[A/V]Lx- motifs were also highly conserved *(Fig. 4B)*.

DISCUSSION

As an infectious pathogen, *P. multocida* has great harm to a wide range of hosts and can cause many diseases. Parallel with the developments on the research of *P. multocida*, it has been almost completely controlled. Nevertheless, the pathogenic mechanism is currently not well understood, and thus it still restricts the development of livestock and poultry breeding and causes severe economic losses [9,33]. In this study, 23 strains of *P. multocida* isolated from poultry in Chongqing and Sichuan, China were tested for capsular antigens and antimicrobial susceptibility. The results showed that the capsular types of isolates were predominantly type A which is similar to that it was usual reported for *P. multocida* in China and other countries [34,35]. But we found one isolate of *P. multocida* was type F which is similar to the findings that reported previously [33]. Meanwhile, in these two provinces, antibiotic therapy is still an effective tool in the treatment of infections caused by *P. multocida*. Similarly, the potential threat of such multi resistant bacteria in food producing animals should not be neglected.

The *toxA* gene encodes dermonecrotic toxin, but the *toxA* gene is not detected in this study. This result may indicate that the avian strain or serotype A of *P. multocida* is not easy to carry the *toxA* gene. Some research suggested that the *toxA* gene is more significantly associated with serotype D, compared to serotype A [33,36]. We investigated iron-regulated and

iron-acquisition related genes *(tonB*, *fur*, *hgbB*, *hgbA)* irrespective of the animal clinical status and found that all isolates had the *tonB* gene similar to the findings reported previously [37]. In a past study [38], the *tonB* gene encoded an energy transfer protein.

TonB protein is a periplasmic protein prevalent in a large number of Gram-negative bacteria, which is believed to be responsible for the import of ferric iron complexes across the outer membrane [39,40]. Not only TonB protein activates outer membrane to make substrates into the periplasm as an energy transduction component [41], but also plays a role as an important virulence factor to help Gram-negative bacteria to obtain nutrients. In this study, we attempted to amplify and clone the *tonB* gene from 23 isolated strains of *P. multocida*, and then to explore the evolutionary relationship of the *P. multocida tonB* genes with different host and serotypes have. It was showed in *Fig. 2*, *P. multocida tonB* gene is present in different hosts and also in different serotypes. The phylogenetic tree displayed that these *tonB* genes have close genetic relationship and there is a high similarity (92.9%-100%) among these *tonB* genes. A similar distribution of certain genes, regardless of the host species of *P. multocida* serotype, may suggest that the selection of factors that present cross-protection as candidates for vaccine development [33,42]. TonB protein is generally conserved among species and has a high immunogenicity. Therefore, TonB protein potentially serves as vaccine candidates.

To study the evolutionary relationship of TonB protein in bacteria, a TonB Hidden Markov Model named TonB-HMM was built. HMMER2.3.2 software was used to retrieve the local protein database based on this model to analyze the genetic characteristics of TonB protein in *P. multocida* and other bacteria. Based on the phylogenetic analysis *(Fig. 3)*, we found that TonB protein of *P. multocida* shared a high homology with TonB protein of *Alphaproteobacteria* and *Gammaproteobacteria* classes in *Proteobacteria* phylum. Multiple sequences alignment was performed and the results reported in *Fig. 4A* show that the -xSSGx- and -YP- motifs were the best conserved portion in these four groups, which is consistent with the data reported previously about the -xSSGx- motif as a highly conserved motif in TonB protein [40]. It is well known that there is a certain functional value may exist in conserved sequences and it was reported that the -xSSGx- motif might play a role in TonB protein and receptor recognition [39]. Previous studies on -YP- motif had emphasized that C-terminal domain of TonB protein was closed to the cobalamin transporter BtuB [43]. In addition, the -YP- motif contacted with ferricytochrome receptor (FhuA) and the TonB-box [44]. Moreover, we found that the -LD- and -xA[A/V]Lx- motifs were also relatively conserved, meanwhile, the cloned strain KY748245 was predicted for point mutation. The results suggest that the amino acid of the -LD- and -xA[A/V]Lx- motifs have strong conservative, therefore, to some extent, the function of TonB protein can be further explored by studying the point mutation of the -LD- and -xA[A/V]Lx- motifs, which is highly significant. Thus, we can attempt to perform point mutation, substitution and destruction of these conserved amino acid and motifs to change the function of TonB protein. However, as conserved amino acid and motifs are changed, we are not sure whether it can make pathogenicity weaken, so further studies are required.

In conclusion, we successfully isolated a total of 23 strains of *P. multocida* from poultry, of which 22 strains were identified as serogroup A and one strain was serogroup F. The research provided information regarding the distribution of virulence genes of *P. multocida* strains isolated from poultry in Chongqing-Sichuan regions, China. The *P. multocida tonB* gene was found a high frequency in this study and it was not specific to a host or a serogroup. The genetic evolutionary relationship with TonB protein of Gram-negative bacteria was elucidated by phylogenetic analysis, and conserved motifs of TonB protein were predicted. We can try to study the performance of these motifs from various perspectives, which may be further extended for functional studies of TonB protein in bacteria and provides a reference for the researches of drug target in *P. multocida*.

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