Research Article

Genetic Diversity of Pilin From Kazakh Isolates of *Moraxella bovoculi*

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Abstract | *Moraxella bovoculi* (*M. bovoculi*) is commonly isolated from clinical cases of infectious bovine keratoconjunctivitis (IBK), however, the exact role of *M. bovoculi* in this disease remains unclear. Experimental *Moraxella bovis* (*M. bovis*) and *M. bovoculi* vaccines to prevent IBK based on recombinant pathogenic factors have been evaluated as alternatives to whole cell vaccines. Pilin is one candidate antigen for *M. bovoculi*, and is a key pathogenic factor necessary for attachment of *M. bovis* to the surface of the eye epithelium. To evaluate *pilA* diversity amongst Kazakh isolates of *M. bovoculi*, the *pilA* gene sequence was determined for 35 isolates of *M. bovoculi*. Ten genotypes (gNA1-gNA10) were identified which were 96.1% to 99.8% identical. It was found that as a result of the replacement of cytosine with thymine at the 97th position of the gNA8 genotype, a stop codon resulted which, if expressed, would have resulted in a truncated PilA. Phylogenetic analyses of the deduced amino acid sequences of PilA from isolates from Kazakhstan, in comparison with previously defined PilA groups in *M. bovoculi*, established that 68% of the Kazakh isolates belonged to group A, 8.6% to groups C and F. It was found that despite these isolates originating from geographically close regions, some of the studied isolates had unique amino acid sequences that were assigned to new M. bovoculi PilA groups designated K (2 isolates), L (2 isolates), and M (1 isolate).

Keywords | *Moraxella bovoculi*, infectious bovine keratoconjunctivitis, *pilin, pilA,* PilA

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INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK), is a highly contagious and infectious disease of the bovine eye characterized by conjunctivitis and ulcerative keratitis that occurs worldwide (Holzhauer and Visser, 2004; Kneipp, 2021). Infectious keratoconjunctivitis also occurs in other domestic (Motha et al., 2003) and wild (Giacometti et al., 2002) species and is generally considered to be a multifactorial disease. The most commonly isolated pathogen from IBK is *Moraxella bovis* (*M. bovis*) (McConnel et al., 2007). The presence of *M. bovoculi* isolates in most eye samples taken from IBK-affected cattle suggests a role for *M. bovoculi* in pathogenesis (Angelos, 2010). The frequency of co-isolation of these two species may indicate that *M. bovoculi* serves as an opportunistic agent in IBK (Loy and Brodersen, 2014). In a recent study using the PCR method, *M. bovoculi* was detected in 75% of the samples of the total number of samples examined (Zheng et al., 2019). Other data on a positive correlation between the clinical signs of IBK and the presence of *M. bovoculi* also indirectly support that this organism is involved in the pathogenesis of IBK (Loy and Brodersen, 2014; Schnee et al., 2015).

Pilin is known to be one of the key pathogenic factors in *M. bovis*, as this protein allows moraxella to "stick" to

the surface of the ocular epithelium (Greene et al., 2001; Lehr et al., 1985; Lepper and Hermans, 1986; Prieto et al., 2003; Ruehl et al., 1993). *M.bovis* pili (classified as type 4) show antigenic and phase variation and are classified into seven groups (A to G) (Moore and Lepper, 1991). These pili are composed of repeating, homologous polypeptide subunits called pilin. Type 4 pilins contain many variable and hypervariable domains that are a source of both structural and antigenic variation between species (Ruehl et al., 1993). Two different types of pilin molecules, called I and Q, have been identified as separate gene products. In vitro experiments have shown that phase variation allows *M.bovis* to switch from one type of pilin expression to another (Ruehl et al., 1993). The rather wide diversity of pilin genes among different *M.bovis* isolates may be the reason for the ineffectiveness of fortified pilin preparations proposed as vaccines against IKC. Sequence heterogeneity of a pilin gene (*pilA*) in *M.bovoculi* was also recently reported, however, diversity amongst deduced PilA sequences was much less in comparison to pilin amino acid sequences in *M. bovis* (Angelos et al., 2021).

That there are comparatively large differences between the amino acid sequences of PilA protein in *M. bovoculi* isolated from the nasopharynx of cattle without IBK symptoms versus isolates from the surface of the eyes from animals with IBK suggests a role for pilin in establishing *M. bovoculi* in different environments on the host.

To further expand our understanding of PilA in *M. bovoculi* and help guide potential development of vaccines to prevent IBK, we determined genetic diversity amongst structural pilin (PilA) sequences encoded by *M. bovoculi* from cattle with clinical signs of IBK from different regions of Kazakhstan.

MATERIALS AND METHODS

For this study we used isolates of *M. bovoculi* that were isolated from cattle from various regions of Kazakhstan during May-August 2021.

Samples of biological material from eyes of cattle with clinical signs of IBK were taken using test tubes with Amies transport medium and plated on Columbia agar with 5% bovine serum and then incubated for 20-24 hours at 37°C. Colonies with characteristic growth for *M. bovoculi* were subcultured and used for primary identification by Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS). To do this, single colonies were suspended in 1 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid (α-HCCA) with 50% acetonitrile and 2.5% trifluoroacetic acid (TFA), and dried in air. The chip with samples deposited on it was placed in a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics). The chip was positioned and calibrated against a calibration standard. The spectra were collected automatically using 40 laser pulses (60 Hz). The analyzed mass/charge range was 2000–20000 Da. The spectra were analyzed using the Bruker MALDI-TOF Biotyper v4.0 software; the minimum score for species identification was 1.8.

Isolates were stored at -80°C in trypticase soy broth with 40% glycerol.

Genomic DNA isolation was performed using QIAamp DNA Mini Kit, Qiagen.

The 16S rRNA gene fragment was amplified using universal primers 8f (5'-agagtttgatcctggctcag-3') and 806R (5'- ggactaccagggtatctaat-3') in a total volume of 30 µl (Srinivasan et al., 2015). The PCR reaction was performed with universal primers. PCR reaction included 2.5 mM of $MgCl_2$, 300 nM of each primer, 200 nM each dNTP, 1X Taq Buffer with KCl (Thermo Scientific™), 1 U Taq DNA Polymerase (Thermo Scientific™, EP0401) and 3 ng DNA. The PCR program was 3 min at 95°C, 30 cycles at 95°C for 30 s, 55°C 30 s and 72°C for 50 s, followed by 72°C for 10 min.

The *pilA* pilin gene was amplified using primers Mbovoc_Pilin_Dn (5-gtggggttacataaatataaaga-3') and Mbovoc_Pilin_Up3 (5'-gattaatcaaaccttcaaacac-3') as described (Angelos et al., 2021).

The efficiency of amplification was evaluated using gel electrophoresis. To determine the nucleotide sequence, PCR products were purified from unbound primers by an enzymatic method using Exonuclease I (Thermo Scientific™) and Shrimp Alkaline Phosphatase (Applied Biosystems™) as described (Werle et al., 1994). The sequencing reaction, using the above reaction primers, was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applide Biosystems) according to the manufacturer's instructions, followed by separation of the fragments on a 3730xlDNAAnalyzer automated genetic analyzer (Applide Biosystems). Nucleotide sequences were analyzed and combined into a common sequence in SeqMan software (DNAStar). Nucleotide sequences of 16S rRNA were identified in BLAST (https://blast.ncbi.nlm.nih.gov/) by homologous sequences with the highest percentage of identity (Boratyn et al., 2013), and determination of percent identity with reference sequences of *M. bovoculi*, *M. bovis*, *M. ovis,* and *M. lacunata* (GeneBank acesion numbers: NR_043583, NR_028668, NR_0286701 and NR_036825, respectively) was calculated in MegAlign (DNAStar).

The nucleotide and amino acid sequence of the pilA gene was used to determine genetic diversity by building phylogenetic trees and percent identity. Percent identity was determined in MegAlign (DNAStar). Phylogenetic trees were built in MEGA X (Kumar et al., 2018). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option) (Kumar et al., 2018). The phylogenetic analysis of deduced amino acid sequences included *PilA* sequences of 10 previously identified *PilA* groups in *M. bovoculi* (Angelos et al., 2021).

RESULTS

As a result of bacteriological studies, 35 bacterial isolates were identified on the basis of phenotypic characteristics as *M. bovoculi*. Using the MALDI-TOF method, all isolates were assigned to the genus Moraxella, however, in 33 isolates the protein profile corresponded to *M. bovoculi*, and in two isolates the protein profiles were identified as *Moraxella_sg_Branhamella ovis*, with a high Score Value (1.7-1.9). Genetic identification by nucleotide sequence analysis of the 16SrRNA gene identified all isolates as *M. bovoculi* (Table 1). The percentage of identity with the NR_043583 sequence of *M. bovoculi* was 99.8-100%, with *M. bovis* - 98.4-98.8%, with *M. ovis* - 95.7-96.1% and with *M. lacunata* - 99.4-99.6%. The isolates were from 4 farms in Kostanay, Akmola and Pavlodar regions (Figure 1).

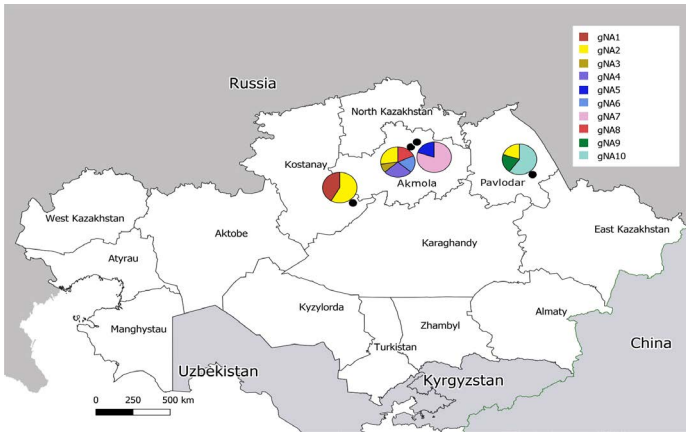

Figure 1: Sample Collection Map

Based on the nucleotide sequence of the *pilA* gene, the analyzed isolates were grouped into 10 genotypes (Figure 2) designated gNA1 through gNA10.

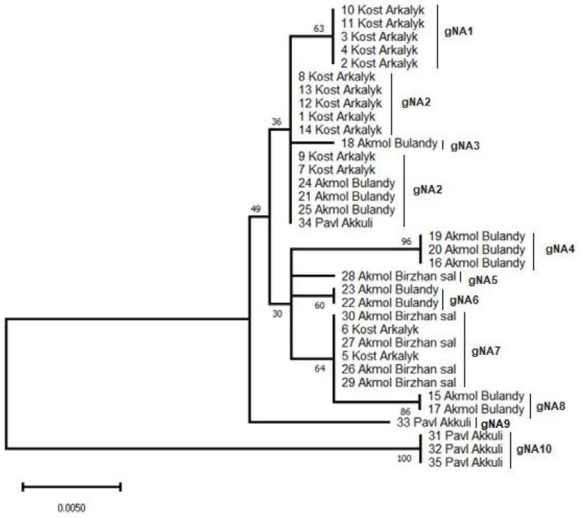

Figure 2: Phylogeny based on the nucleotide sequence of the *pilA* gene of 35 *M.bovoculi* isolates

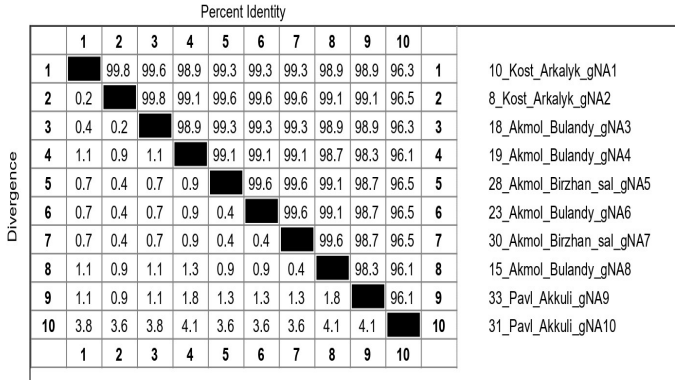

Figure 3: Identity between genotypes of the *PilA* gene.

It was determined that 3 genotypes were represented by single isolates (gNA3, gNA5, gNA9) which were isolated from Akmola and Pavlodar regions. The gNA1 genotype contained 5 isolates from the Kostanay region. The largest gNA2 genotype represented 11 isolates: 7 were isolated on a farm in the city of Arkalyk, Kostanay region, 3 from the Bulandy district of the Akmola region, and 1 from the Akkuli district of the Pavlodar region. The gNA4 genotype includes 3 isolates from the Bulandy district of the Akmola region. The gNA6 genotype was represented by two isolates from the Bulandy district of the Akmola region, which, based on MALDI-TOF, had been identified as *Branhamella ovis*. The gNA7 genotype included 4 isolates from the Birzhan sal district of the Akmola region and 2 isolates from the Kostanay region. The gNA8 genotype contained 2 isolates from the Bulandy district of the

Table 1: Results of isolate identification

Sample number	Region	Date of sampling	Breed of cattle	Identification result	
				MALDI-TOF)	16S rRNA
1	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
2	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
3	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
4	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
5	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
6	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
7	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
8	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
9	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
10	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
11	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
12	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
13	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
14	Kostanay region, Arkalyk	11.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
15	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
16	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
17	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
18	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
19	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
20	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Branhamella ovis*	*Moraxella bovoculi*
21	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
22	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
23	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Branhamella ovis*	*Moraxella bovoculi*
24	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	Moraxella_sg_Branhamella ovis	*Moraxella bovoculi*

25	Akmola region, Bulandy district	31.05.21	Kazakh white-headed	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
26	Akmola region, Birzhan sal district	9.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
27	Akmola region, Birzhan sal district	9.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
28	Akmola region, Birzhan sal district	9.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
29	Akmola region, Birzhan sal district	9.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
30	Akmola region, Birzhan sal district	9.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
31	Pavlodar region, Akkuli district	19.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
32	Pavlodar region, Akkuli district	19.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
33	Pavlodar region, Akkuli district	19.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
34	Pavlodar region, Akkuli district	19.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*
35	Pavlodar region, Akkuli district	19.08.21	Angus	*Moraxella_sg_Moraxella bovoculi*	*Moraxella bovoculi*

Table 2: Amino acid substitutions identified in the PilA protein

Isolate	**Position**						
	33	**61**	**62**	**81**	**98**	**130**	**131**
10 Kost Arkalyk gNA1	R	S	N	V	I	G	Q
8 Kost Arkalyk gNA2	R	S	N	V	I	G	Q
18 Akmol Bulandy gNA3	R	S	N	V	I	G	Q
19 Akmol Bulandy gNA4	R	N	N	V	I	G	Q
28 Akmol Birzhan sal gNA5	R	S	N	V	I	G	Q
23 Akmol Bulandy gNA6	R	S	N	V	V	G	Q
30 Akmol Birzhan sal gNA7	R	S	N	V	I	G	Q
15 Akmol Bulandy gNA8	*	S	N	V	I	S	Q
33 Pavl Akkuli gNA9	R	S	N	V	I	D	K
31 Pavl Akkuli gNA10	R	S	S	P	I	G	Q

Akmola region. The gNA10 genotype was represented by 3 isolates from the Pavlodar region. Identity between genotypes varied from 99.8% to 96.1%, which, in nucleotide equivalents, was from 1 to 17 nucleotides (Figure 3).

Most of the variable nucleotides were silent mutations and did not lead to protein amino acid substitutions. A total of 8 nucleotides were missense mutations and resulted in amino acid substitutions (Table 2). The maximum difference between the genogroups was 2 amino acid residues out of 152.

The deduced PilA amino acid sequence from genotypes gNA1, gNA2, gNA3, gNA5 and gNA7 did not differ from each other. In the gNA4 genotype, serine was replaced by asparagine at position 61 (S61N). In the gNA6 genotype, at position 98 of the amino acid sequence, isoleucine was replaced by valine (I98V). In the gNA8 genotype, the substitution of cytosine for thymine at position 97 of the nucleotide sequence of the *pilA* gene led to the formation of a stop codon, which would result in the expression of a truncated protein in the 15_Akmol_Bulandy and 17_Akmol_Bulandy isolates. Also in this genotype, a substitution of glycine for serine at position 130 (G130S) was identified. In the gNA9 genotype, 2 amino acid substitutions were found at positions 130 and 131 of glycine for aspartic acid and glutamine for lysine, respectively (G130D and Q131K). In the gNA10 genotype, two amino acid substitutions of aspargine for serine at position 62 of the protein and valine for proline (N62S and V81P) were also found.

Phylogenetic analysis of the PilA deduced amino acid sequence of isolates from Kazakhstan with previously established groups made it possible to establish the group affiliation of the PilA gene genotypes (Figure 4). It was determined that more than 68% of the isolates (N=24) belonged to PilA group A. Three isolates each belonged to PilA groups C and F. Two isolates from the Akmola region under accession numbers 22 and 23 have a unique amino acid sequence and were assigned to a new group designated PilA group K. The two truncated PilA sequences (gNA8; isolates 15 and 17) were located in a separate clade and were assigned to an additional new group designated PilA group L. Isolate number 33 was also located separately and was designated as belonging to a new group designated PilA group M.

Nucleotide sequences of pilA gene has been deposited in GenBank under accession numbers OP352320 - OP352329.

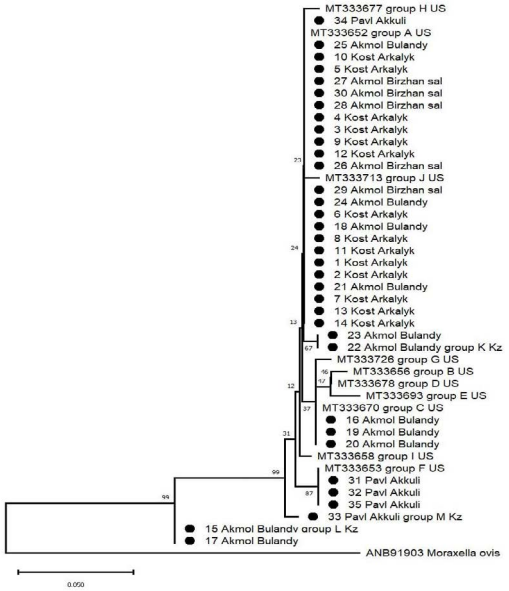

Figure 4: Phylogenetic tree based on the amino acid sequence of the *PilA* gene. The *Moraxella ovis* sequence was used to root the tree.

DISCUSSION

According to the conclusion of a number of researchers, IBK is one of the infections that cause the greatest economic damage to livestock (Brown et al., 1998; Prieto et al., 2013; Postma et al., 2008; Dennis et al., 2021). In the last decade in Kazakhstan, the number of cases of IBK has increased, which are primarily associated with the import of highly productive animals, the infection rate reaches 27% (Ivanov et al., 2021). Over the past few decades, numerous studies have been carried out to study the effectiveness of various types of vaccines for the prevention of IBK. In most cases, the emphasis was on whole-cell bacterial *M bovis/M. bovoculi* preparations and pilin antigens. The current lack of universally effective IBK vaccines suggests that further research is needed on pathogenicity and immunogenicity of *Moraxella* spp associated with IBK in cattle (Hille et al., 2022; Funk et al., 2009; O'Connor et al., 2019). The identification of conserved nucleotide sequences of genes encoding proteins with immunogenic potential is an important step towards creating improved vaccine preparations (Acquistapace et al., 2021). The data presented here should help guide future studies aimed at identifying more effective pilin-based vaccines to prevent IBK.

This is the first study in Kazakhstan to study the genetic homogeneity of *M. bovoculi* genes associated with IBK. Whether or not the differences that we identified in deduced PilA sequences correspond to differences in pathogenic potential of *M. bovoculi* remains to be determined. Further studies that examine temporal changes in PilA group circulating in herds of cattle may be needed to help answer this question.

Genetic identification showed that all 35 isolates from cattle in the farms of Kostanay, Akmola and Pavlodar regions were *M. bovoculi*. Some researchers suggest the lack of pigmentation around the eyes in animals of some breeds (Hereford and others) as one of the predisposing factors of IBK (Ward and Nielson, 1979). In this study, *M. bovoculi* isolates were from cattle with clinical signs of IBK of the Kazakh Whitehead (lack of pigmentation) as well as Angus breeds. Thus, in the cattle from which these isolates were derived, we did not observe any clear association between IBK and pigmentation.

As a result of the sequencing of the *pilA* gene sequences of the *M. bovoculi* isolates, it was determined that they belonged to 10 different genotypes, the identity between which varied from 96.1-99.8%. Due to the replacement of cytosine with thymine in the 97th position of the gNA8 genotype, a stop codon was formed, which would result in expression of a defective PilA protein in two isolates. PilA has been identified as a major virulence factor in some Gram-negative bacteria (Forslund et al., 2010), however, one previous study reported no effect on the pathogenicity of Pseudomonas aeruginosa isolates by turning off the pilA gene (Choi et al., 2002). Phylogenetic analysis of the amino acid sequence of PilA from *M. bovoculi* isolates from Kazakhstan, with previously established groups, made it possible to determine that 68% of Kazakh isolates belonged to group A, which is consistent with previous studies indicating a high prevalence of this group amongst strains isolated in the USA (Angelos et al., 2021). Groups C and F included 8.6% of isolates. Some of the studied isolates had a unique amino acid sequence and can be assigned to 3 additional PilA groups, K, L, and M.

In general, there is a high degree of homogeneity in the deduced amino acid sequence between the isolates included in the 13 established groups A-L.

More extensive and systematic studies will be needed to determine if any attempts by *M. bovoculi* to evade the host's immune system is reflective of differences in PilA sequences.

CONCLUSION

The *pilA* gene from Kazakh isolates of *M. bovoculi* had a high degree of genetic homogeneity. PilA may be a worthwhile vaccine antigen to consider when creating vaccines to prevent IBK. Nevertheless, the identification of a putative truncated PilA in two isolates from animals with IBK requires further investigation of its role in pathogenesis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

NOVELTY STATEMENT

Sequencing of pilA in Kazakh isolates of M. bovoculi revealed 3 novel PilA deduced amino acid sequences. Additional research is needed to determine if PilA is involved in the biology of M. bovoculi as it relates to the presence of this organism in eyes of cattle with infectious bovine keratoconjunctivitis.

AUTHORS CONTRIBUTION

This manuscript's materials preparation, data analysis, and text writing were all done by all of the authors.

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