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Serological examination of *Mannheimia haemolytica* and *Bibersteinia trehalosi* in cattle

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List of abbreviations

BRD	Bovine Respiratory Disease
BRDC	Bovine Respiratory Disease Complex
M. haemolytica	*Mannheimia haemolytica*
B. trehalosi	*Biberstenia trehalosi*
M. glucosida	*Mannheimia glucosida*
H. somni	*Histophilus somni*
IHA	Indirect haemagglutination test
q-PCR	Quantitative polymerase chain reaction
RCF	Relative centrifugal force
PCR	Polymerase chain reaction
NT	Non typable
RPM	Revolutions per minute
LKT	Leukotoxin
RSV	Respiratory syncytial virus
PI3	Parainfluenza virus 3
BVD	Bovine viral diarrhoea

Introduction

Mannheimia haemolytica, formerly known as *Pasteurella haemolytica* is one of the primary bacterial pathogens associated with bovine respiratory disease (BRD) in cattle worldwide, resulting in significant economic losses.
M. haemolytica typically resides as a commensal in the nasopharynx of healthy ruminants. However, it can infiltrate the lower respiratory tract when the host's defenses are compromised due to a variety of production management or environmental stressors, along with coinciding viral infections.
Bibersteinia trehalosi is primarily identified as a pathogen of sheep causing septicaemia or pneumonia. *B. trehalosi* is considered rare in cattle and limited data has been published on this however, there has been an increased interest in it recently as severe BRD cases associated with *B. trehalosi* showing multi drug resistance have been recorded [1].

Administering antimicrobials for both the therapeutic and prophylactic treatment of BRDC is the solution in many cases however, the increase in concerns of antimicrobial resistance has created a growing interest in the use of vaccines against BRDC. Vaccines have been available for the past few years to prevent BRDC but unfortunately, they have not been entirely successful as outlined in Bowland and Shewens study in 2000 as it is a multifactorial disease and often the vaccine only targets *M. haemolytica* serotype A1 [2]. Identification of the prevalent serotypes is imperative in the control of *M. haemolytica* and *B. trehalosi* infection and provides important information for the development of vaccines.

There have been few studies carried out in relation to the prevalence of *M. haemolytica* and *B. trehalosi* serotypes in Europe [3], but these have focused on the prevalence and antimicrobial susceptibility rather than the serotype [4,5]. Examining the different serotypes prevalent in a region is critical in ensuring that vaccines are covering the serotypes causing the disease. Therefore, the purpose of this study is to assess the prevalence of *M. haemolytica* and *B. trehalosi* serotypes associated with BRDC clinical signs in Hungarian cattle.

Literature Review

1. Mannheimia haemolytica

Mannheimia haemolytica, previously known as *Pasteurella haemolytica* is typically associated with pneumonic pasteurellosis in cattle and sheep, septicaemia in lambs and mastitis in ewes [6]. *M. haemolytica* is one of the dominant bacterial pathogens linked to bovine respiratory disease (BRDC), and shipping fever. The complex of BRD leads to significant economic setbacks in the cattle sector due to expenses related to treatment, reduced production, handling, and instances of mortality [7].

M. haemolytica are small gram-negative coccobacillus displaying slight pleomorphism and occasional bipolar staining [8]. *M. haemolytica* strains were originally categorised into two biotypes, according to arabinose and trehalose utilisation, positive strains were marked with A and T. Based on DNA findings, *M. haemolytica* underwent a reclassification leading to three genetically distinct species: serotypes A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16 and A17 were described as *M. haemolytica*, serotypes T3, T4, T10 and T15 were described as *Biberstenia trehalosi* and serotype A11 was described as *Mannheimia glucosida* [9, 10, 11]. Each isolate is identified by a combination of a letter and number indicating the serotype and biotype. Figure 1 provides a summary of the serotype classification.

These species can be serotyped according to their capsular antigens. Leukotoxin is the primary virulence factor of *M. haemolytica* and *B. trehalosi*. The occurrence of beta-hemolysis on blood agar is linked to the presence of the leukotoxin gene, although it does not provide definitive confirmation [12, 13].

Figure 1. Summary table of species and their corresponding serotypes.

SPECIES	SEROTYPE
M. HAEMOLYTICA	A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, A17
M. GLUCOSIDA	A11
B. TREHALOSI	T3, T4, T10, T15

Amongst healthy cattle, *M. haemolytica* typically resides in low numbers within the nasopharynx and tonsils, making it challenging to identify through nasal swab cultures [14]. Following the findings of Rice and his team in 2007, it is proposed that *M. haemolytica* establishes a commensal relationship with the host until circumstances alter due to stress or concurrent infection [15]. The exact mechanism by which environmental stressors or viral infections, whether separately or in combination, increase the susceptibility of cattle to developing shipping fever are not known. Once this harmonious relationship is disturbed, the A1 serotype swiftly takes over as the dominant organism and is accountable for the characteristic bronchopneumonia.

A limited number of studies have identified the specific *M. haemolytica* serotypes linked to respiratory disease in cattle [16, 17, 18, 19, 20]. It is clear from these studies that the proportion of strains differs from region to region. Serotype A1 strains are most frequently associated with lungs affected by pneumonia although serotype A6 strains are increasingly responsible for a growing proportion of disease cases, while serotype 2 is mainly associated with healthy asymptomatic cattle [3, 7]. Fatalities can occur in two to three days, or the infection can progress leading to lasting lung impairment. The initial clinical signs of the disease include fever, cough, lethargy, anorexia, dyspnoea as well as discharge from the nose and eyes [14].

The last published serological examination of *M. haemolytica* and *B. trehalosi* in Hungarian cattle was undertaken by Fodor et al. in 1999. The strains were isolated from the carcasses of deteriorated animals who underwent post-mortem examination or from the discharges of animals who presented with respiratory clinical signs. Serotypes isolated were mainly A1 (59.4%, 69/116) and A2 (24.1%, 28/116). Fodor found that serotype A1 was the most frequently isolated serotype from the lungs of cattle. Serotype A2 was primarily isolated from nasal discharge (15/28) but was also found in the lung (10/28) and the prepuce (1/28). The majority of the non typable strains were isolated from extrapulmonary

sources [16]. Figure 2 outlines the serotypes and their anatomical origins from this study.

Figure 1. Serotypes isolated and anatomical origins

	Lung	Nasal discharge	Trachea	Conjunctiva	Spleen	Navel	Fetus	Foetal membranes	Vagina	Prepuce	Total	%
A1	38	28	-	1	2	-	-	-	-	-	69	59.4
A2	10	15	2	-	-	-	-	-	-	1	28	24.1
A7	3	-	-	-	-	-	-	-	-	-	3	2.6
A8	-	-	-	-	-	-	1	-	-	-	1	0.9
A9	1	-	-	-	-	-	-	-	-	-	1	0.9
T10	1	-	-	-	-	-	-	-	-	-	1	0.9
A16	-	4	-	-	-	-	-	-	-	-	4	3.4
NT	2	2	-	-	-	1	1	2	1	-	9	7.8
Total	54	50	2	1	2	1	2	2	1	1	116	100

In a thesis conducted in 2014, 23 strains were obtained from Hungarian cattle displaying clinical signs of BRDC, originating from 14 distinct livestock sources spanning the years 2010 to 2014. Serotyping was performed using the passive hemagglutination method (IHA), revealing a notable prevalence of A2 strains (11/23, 47.8%) compared with serotype A1 which accounted for 13% of the isolates (3/23). The majority of A2 serotypes were isolated from the nose (6/11, 54.5%) while 5/11 (44.5%) isolates were found in the lungs. Additionally, M. glucosida was isolated from the lungs on one occasion, an infrequent finding in cattle [21]. Figure 3 summarises the serotypes isolated in this study.

Figure 3. Results of Dóra Szalay's thesis investigation

Serotype	No. of strains in cattle	%
A1	3	13
A2	11	47.8
A5	1	4.3
A7	1	4.3
A11	1	4.3
T15	1	4.3
NT	5	21.7
SUM	23	100

A recent serological examination of *M. haemolytica* from European cattle with BRD described prevalence of serotypes A1 (59%) and A6 (22%) while A2 was represented by 18% of the isolates [19]. This result correlates with others in the fact that A1 is the most common serotype in clinical BRD cases [3, 16, 18, 20]. Arnal et al. (2021) underlines the fact that although the serotype A2 is commonly believed to be isolated from the respiratory systems of healthy animals, their results correlate with those of other clinically ill animals in which serotype A2 represented a remarkable percentage of the serotypes isolated [16, 18, 19]. Thus, it is with great caution that this idea be accepted.

According to recent literature there is undoubtably an increased isolation of serotype A6 in cattle across the UK, Germany and Japan [18, 22]. This finding

contrasts with previous studies undertaken during the 1900s in Hungary and Denmark where A6 was not isolated [3, 16]. A comprehensive study carried out by Katsusu et al. (2008) in Japan, describes how over the course of two decades, a total of 207 *M. haemolytica* samples were isolated by indirect hemagglutination from pneumonic calves. From the 1980s through to the early 1990s, serotype A6 was not isolated from cattle however, from the late 1990s the number of times serotype A6 was isolated increased from 5 times to 15 times, underlining the significance of noting this emerging strain to aid with the production of efficacious vaccines in Japan [18].

2. Mannheimia glucosida

Serotype A11 encompasses all typable strains of *M. glucosida*. This species is phenotypically heterogenous, but all strains show beta- glucosidase activity [10]. This group of bacteria is typically unrelated to disease conditions and likely constitutes a component of the normal resident microflora in the upper respiratory tract [23]. *M. glucosida* is commonly isolated from ovine animals however, this serotype has been identified in cattle also but not as frequent as *M. haemolyticia* [24, 25, 26]. None of the published studies examined in this literature review have isolated this serotype, enforcing the fact that either it is infrequently isolated from cattle or perhaps despite new species being outlined, the accurate identification of isolates remains challenging and labour intensive. Figure 4 summarises the *M. haemolytica* serotypes isolated in the cited studies.

A study describing a PCR assay specialised for the identification of *M. haemolytica, M. glucosida* and *M. ruminalis* described the importance of using PCR techniques in order to improve efficacy in identifying M. glucosida, instead of being mistakenly identified as *M. haemolytica* [28].

Figure 4. Summary of *M. haemolytica* serotypes isolated from cattle

	VILLIARD ET AL. (2006)	**KATSUDA ET AL. (2008)**	**ANDRES LASHERAS ET AL. (2019)**	**ARNAL ET AL. (2021)**
A1	-	102/207, 49.3%	48/80, 60%	28/54, 51.8%
A2	3/4, 75%	47/207, 22.7%	16/80, 20%	9/54, 16.7%
A5	-	-	-	1/54, 1.9%
A6	-	42/207, 20.3%	16/80, 20%	4/54, 7.4%
A7	-	-	-	1/54, 1.9%
A12	1/4, 25%	-	-	-
A16	-	-	-	2/54, 3.7%
NT	n/a	13/207, 6.2%	n/a	9/54, 16.7%

3. *Bibersteinia trehalosi*

Bibersteinia trehalosi has long been recognized as a pivotal pathogen in sheep, primarily associated with septicaemia in lambs between 4 and 10 months old and pneumonia [11]. Studies have been published in which *B. trehalosi* has been isolated from other ruminant species including cattle, goats and it is currently the most prevalent organism of the Pasteurellaceae family to be cultured from the tonsils of clinically healthy American bison (Bison bison) [28].

B. trehalosi is a member of the Pasteuellaceae family and is characterised as a small gram negative, nonmotile, aerobic and facultatively anaerobic pleomorphic rod. It can be observed singly, in a short chain or in pairs [11]. In 1959, *Bibersteinia trehalosi* was initially identified as *Pasteurella haemolytica* biotype T, and it was later reclassified as *Pasteurella trehalosi* in 1990 [10, 11]. There are four distinct capsular serotypes – T3, T4, T10, T15, which were first described by Biberstein et al. (1960). In honour of Biberstein work, the organism

was renamed *Bibersteinia trehalosi* during 2007 [29]. These two organisms are indistinguishable on gross postmortem examination or routine culture as they as closely related [10]. An additional test involving sugar digestion is necessary to distinguish it from *M. haemolytica* as *B. trehalosi* can digest trehalose unlike *M. haemolytica*.

While it is the primary bacterial pathogen in sheep causing fatal pneumonia and septicaemia, *B. trehalosi* is infrequently identified as a respiratory pathogen in cattle. Initial reports of clinically significant *B. trehalosi* infections in cattle originated in Europe. Wray and Thompson (1957) identified serotype T3 from the pneumonic lungs of a calf on one occasion [30]. In 1985, Alan et al. (1985) isolated serotype T10 four times from cattle on two different farms which were suffering from an outbreak of a respiratory disease [31].

The last major serological examination of B. trehalosi in Hungarian cattle was undertaken by Fodor et al. in 1999. The strains were isolated from the carcasses of deteriorated animals who underwent post-mortem examination or from the discharges of animals who presented with respiratory clinical signs. Only one *B. trehalosi* serotype (T10) was isolated from the lungs, whereas Szalays's thesis investigation revealed the isolation of a different serotype, T15 [16, 21]. Although the isolation of B. trehalosi species may be infrequent, its presence is notably discernible within past Hungarian serotyping investigations.

B. trehalosi has been isolated from the respiratory tract of both healthy and diseased animals in the United Kingdom since 2003, and it is believed that cattle of all ages exhibit susceptibility [32]. The isolation of *B. trehalosi* from clinically diseased bovine animals is becoming more frequent with a publication from the United Kingdom describing *B. trehalosi* as the sole pathogenic organism detected in 28% of pneumonic cattle [32].

In 2012, Cortese et al. described numerous cases involving a fatal pneumonia in cattle in America in which *B. trehalosi* was the primary and often the only pathogen isolated. The cases were observed in a group of lactating dairy cows, feedlot bulls and a group of calves, indicating the wide age range of bovine

animals susceptible to this organism. In every case, approximately 10% of the group experienced respiratory disease, which frequently led to fatality. Post-mortem examinations revealed that 50-75% of the lungs in each animal were affected, often showing signs of fibrinous bronchopneumonia. This study states that pure cultures of *B. trehalosi* were identified but the exact serotypes were not described. Despite administering aggressive antimicrobial treatment, little to no improvement was noted [33].

According to Collins (2011) it was deduced that *B. trehalosi* is indeed a potential bacterial component of BRDC and this theory is supported by a recent study carried out by Brown and his colleagues in 2021 [34]. He describes an acute fibrinous pleuropneumonia and septicaemia caused by *B. trehalosi* in neonatal calves in New Zealand. This is the first study published describing the clinical findings and histological lesions of *B. trehalosi* pleuropneumonia of calves in New Zealand. Fifteen neonatal calves out of a group of forty collapsed suddenly with respiratory symptoms. All calves received adequate good quality colostrum and their umbilicals were sprayed with iodine after arrival to housing. On postmortem investigation of the affected calves, prominent pulmonary interlobular oedema was evident and the absence of the typical histological lesions of *M. haemolytica* infection were noted. No other bacteria were detected on microbial culture of the lung joint or peritoneal swab, and the response of four calves to treatment with cefquinome ruled out a viral co-aetiology. It was concluded that *B. trehalosi* was likely to act as a primary pathogen in acute fibrinous pleuropneumonia and septicaemia in calves in New Zealand [34]. This further highlights the increasing significance of this organism in the role of respiratory conditions in bovine animals.

In contrary, many studies have also described the infrequency of *B. trehalosi* isolates from cattle suffering from BRDC, reinforcing the idea that this organism is rare in cattle and does not play a role in causing respiratory disease [35]. In addition to this, Hanthorn et al (2014) studied the pathogenicity of *B. trehalosi* in respiratory disease among calves. Thirty-six calves were inoculated with *B. trehalosi* leukotoxin intra-tracheally and a necropsy was undertaken ten days later. Calves inoculated with *B. trehalosi* did not exhibit increased lung

involvement when compared to the control group of calves. This study contradicts the idea that *B. trehalosi* is an important pathogenic organism in the development of BRDC in cattle but supports the idea that it plays a role as a secondary or opportunistic bacterium. However, it is important to keep in mind the small sample size involved along with the questionable inoculation method and the potential previous exposure of the calves tested to *B. trehalosi,* which could have provided an immunological response [36]. As outlined by Blackall et al. (2007) and Brown et al. (2021), it is possible that the low incidence of the isolation of this organism from cattle in the past could be as a result of the close similarities it shares with *M. haemolytica*, possibly resulting in a misdiagnosis [11, 34].

4. Possibilities of serotype determination

Indirect hemagglutination (IHA) is a common method used to serotype *M. haemolytica* and *B. trehalosi* [37]. Disadvantages of this method described by Arnal et al. (2021) include the possibility of cross reactions due to the use of polyclonal sera. In addition to this, the analysis of serotypes can be subjective to each individual [20].

PCR assays offer a highly convenient and dependable approach for serotyping, as opposed to the conventional serotyping method, which is considerably slower and demands the production and preservation of an extensive collection of hyperimmune sera, a process known to be challenging [38]. In the past, a PCR assay designed for *M. haemolytica* detection was unable to differentiate *M. glucosida* [39]. Furthermore, an investigation revealed that the primers intended for leukotoxin (lkt) amplification not only identified *M. haemolytica's* lkt but also that of other *Mannheimia* species, including *M. glucosida* and *M. ruminalis*. [40].

Killion et al. (2018) developed a real-time PCR assay which demonstrated specificity for the leukotoxin gene in *B. trehalosi*, offering a quick and direct means to identify leukotoxin-producing variants of this bacterium in samples

containing a mixture of leukotoxin-positive Pasteurellaceae species [41]. Both Arnal et al. (2021) and Andrés-Lasheras et al. (2019) compared the results of both a commercial q-PCR [42] with IHA in determining the serotypes of *M. haemolytica* A1, A2 and A6. Almost complete agreement was achieved for serotypes A1 and A2 and significant agreement for A6. It is described as a valuable tool which is an appropriate replacement to IHA to identify the common serotypes of A1, A2 and A6 in cattle [19, 20]. Andrés-Lasheras et al. (2019) suggests the commercialisation of this q-PCR assay in order to aid the surveillance of *M. haemolytica* on a regional basis, enhancing our understanding of serotype distribution. However, while this PCR assay represents progress, it falls short in detecting new serotypes, a capability that the IHA method possesses.

While PCR techniques hold immense significance, accurate identification of serotypes of these bacteria primarily rely on bacteriological examination, biochemical characteristics and, biotyping and serotyping of the isolates. It is important to emphasize that the detection of new serotypes continues to be attainable only through IHA. The identification of less common serotypes similarly remains a distinct feature of IHA.

5. Vaccination

In recent years, there has been a growing availability of vaccines against BRD on the European market and ensuring ample access to these vaccines is a top priority in the European Union [43]. Along with decreasing the economic burden of BRDC and improving animal wellbeing, an additional potential advantage of successful vaccination is a decrease in antimicrobial use and a reduction in the emergence of antimicrobial resistance.

The effectiveness of vaccines against BRD is poorly illustrated. Research has been conducted over several decades on the effectiveness of vaccines against the common bacterial pathogens of BRD, but this research provides limited evidence that the use of vaccines against BRD pathogens is successful [44, 45,

46]. Typically, the effectiveness of these vaccines is evaluated by measuring the humeral and/or the cellular immune response against the infectious agent in question under laboratory [47, 48] or field conditions [49].

There are several vaccines targeting pneumonia on the market. *Biobos respi* 3 and *4* is produced by Bioveta and is a multivalent vaccine targeting the common viruses associated with pneumonia, and it also contains the inactivated serotype A1 of *M. haemolytica* [50]. *Hiprabovis pneumos* vaccine contains inactivated *M. haemolytica* A1 and leucotoxoid and inactivated Hisophilus somni [51]. Two of the most common vaccines against BRD available in Europe at the moment include *Bovilis Boviast RSP (Bovipast*) produced by MDS animal health and *Bovalto Respi 3 (Bovalto*) produced by Boehringer Ingelheim [53, 54]. These are multivalent vaccines targeting *M. haemolytica* serotype A1 as well as BRSV and PI3. The vaccine integrates iron regulatory proteins' technology into its formulation. Animals who receive the vaccine generate antibodies against these proteins, effectively depriving the bacteria of a vital nutrient iron [54]. According to Bovipast, cross reactive immunity with serotype A1 and serotype A6 has been demonstrated under laboratory conditions [53]. This cross protection was also demonstrated by Crouch and his colleagues when calves who were vaccinated with *M. haemolytica* serotype A1 were experimentally challenged with serotype A6. The vaccinated calves that received the vaccination displayed milder clinical symptoms of respiratory disease and thus exhibited reduced lung lesion scores compared to the control group [55].

A field study undertaken by Makoschey et al. (2008) examined the efficacy of the inactivated vaccine *Bovilis Bovipast* containing the iron regulated proteins from *M. haemolytica*. There were three groups- the live vaccine, the inactivated, and the control group. The live vaccine did not contain any bacterial proteins. *M. haemolytica* was isolated from a number of animals vaccinated with the live vaccine or unvaccinated group but not from the group who received the inactivated vaccine containing the *M. haemolytica* proteins [56].

Another field study carried out by Berge et al. (2021) evaluated the humoral response in calves vaccinated with respiratory pathogens. They assessed the two most common European BRD vaccines currently on the market- Bovilis Boviast RSP (Bovipast) and Bovalto Respi 3 (Bovalto). *M. haemolytica* titres were evaluated using a commercial enzyme linked immunosorbent assay (ELISA) test. The calves who had received the Bovipast vaccines had remarkedly higher ELISA titres compared to the Bovalto calves and the negative control calves. The calves who had received Bovalto vaccines had similar Elisa titres to the unvaccinated calves [57]. The poor efficacy of the Bovalto vaccine may be due to the presence of inhibiting maternal antibodies however, this study highlights the remarkable difference in humoral immunity despite the vaccines containing the same antigens targeting the same pathogens. Figure 5 outlines the available commercial vaccines in Hungary and the bacterial and viral antigens they target.

A vaccine that is designed to specifically target *B. trehalosi* in cattle does not exist. Despite both *M. haemolytica* and *B. trehalosi* belonging to a separate genus and species, they share certain lipopolysaccharides and leukotoxin [36, 58]. Consequently, *M. haemolytica* containing vaccines has shown cross protection with B. trehalosi due to the high concentration of leukotoxoid and cell wall antigen [33, 58].

Vaccines are available; however, their effectiveness remains questionable. This can be owed to the fact that the surface antigens of *M. haemolytica* differ among serotypes, making cross protection very challenging. A limited number of studies examine this cross protection and only one commercial vaccine claims to exhibit cross protection. However, this cross protection was exhibited under laboratory conditions further underling the need of undertaking future experiments under field conditions rather than using experimental challenges, thereby allowing the natural exposure of pathogens [59].

An autovaccine, also known as an autogenous vaccine, is a customized vaccine produced for a specific group of animals, often on a single farm or in a particular geographic area. These vaccines are created using pathogens or antigens isolated

from the animals within that specific group to combat diseases or infections unique to that group. Autovaccines are tailored to the specific needs of a particular population and are not commercially available or widely used. Autovaccines come into play when conventional commercial vaccines prove ineffective against a particular disease outbreak. In Hungary, these specialized autovaccines can be obtained through autovakcina.hu [60]. While commercial vaccines offer widespread accessibility and cost-effectiveness, they may fall short in terms of specificity for emerging or locally prevalent strains. This underscores the significance of turning to autovaccines for precision in localized disease management.

Figure 5. Summary of available commercial vaccines against *M. haemolytica* and their respective target antigens in Hungary

	RSV	**BPI3**	**BVD**	***H. SOMNI***	***M. HAEMOLYTICA* SEROTYPE A1**
BIOBOS RESPI 3	X	X			X
BIOBOS RESPI 4	X	X	X		X
HIPRABOVIS PNEUMOS				X	X
BOVILLIS BOVIPAST	X	X			X
BOVALTO RESPI 3	X	X			X

Aims

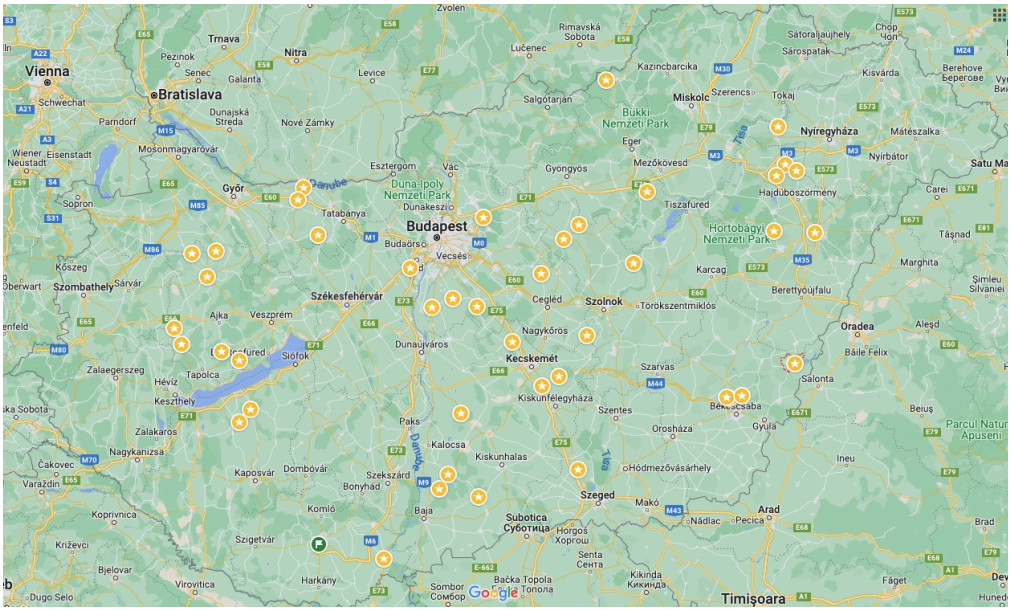
Identifying the serotypes associated with respiratory disease in cattle is crucial for gaining a precise understanding of the epidemiology of *M. haemolytica* and *B. trehalosi* infections. Owing to the fact that immunity against these bacteria is serotype-specific, accurate serotyping is essential for effective vaccination.
This study was therefore conducted to examine the prevalence of the different serotypes of *M. haemolytica* and *B. trehalosi* in Hungarian cattle experiencing BRDC clinical signs.

Materials and Methods

Source of strains

A total of 72 *M. haemolytica*, *2 M. glucosida* and 8 *B. trehalosi* strains were isolated from cattle between 2017 and 2023. Isolates were collected from random animals who presented with clinical BRD signs from the upper respiratory tract or the lower respiratory tract, depending on the type of sample available. In three cases, extrapulmonary samples were taken from the spleen and the blood. The samples were received from a total of forty-two different settlements in Hungary. Figure 6 illustrates the uniform distribution of settlements from which the isolates originated. The exact origin in Hungary was not known for 13 samples. On average, two samples were received from each settlement with the maximum being six samples from the same settlement. Each strain was placed in a cyro tube, by mixing a twenty-four-hour culture of an agar plate of bacteria, 700µl Mueller-Hinton broth and 300µl glycerol. The strains were stored at -80°C.

Figure 6. Map of Hungary showing distribution of isolates collected



Biochemical and serological examinations

Using the indirect haemagglutination test (IHA) described by Biberstein, the *M. haemolytica* and *B. trehalosi* strains were serotyped [23]. The primary materials used included purified red blood cells obtained from defibrinated sterile sheep blood, labelled hyperimmune sera for the assay, and the bacterium strains being analysed. The hyperimmune sera used was produced in rabbits against the strains *M. haemolytica* and *B. trehalosi*. Figure 7 details the origin of the reference strains used.

Figure 7. Origin of Reference strains.

Czech Collection of Microorganisms (Brno, Czech Republic)	*M. haemolytica* A1, A2, A5-A9, A12 *M. glucosida* A11 *B. trehalosi* T3, T4, T10
Moredun Research Institute (Edinburgh, United Kingdom)	*M. haemolytica* A13, A14 *B. trehalosi* T15
ÁTE, Dept. of Epidemiology and Microbiology (Hungary)	*M. haemolytica* A16
Universität Hohenheim (Germany)	*M. haemolytica* A17

Production of hyperimmune sera

Female New Zealand white rabbits were used for the production of hyperimmune sera. The rabbits were aged between 3 and 6 months old and were clinically healthy. They resided in individual cages.

In order to prepare the inoculum, 24-hour old pure cultures of the type strains were collected from the bacteria in the plate and mixed with 20ml Salsol infusion and homogenised. Two weeks prior to the inoculation, all rabbits were monitored, and each rabbit received an individual marking. Figure 8 outlines the inoculation regime.

Figure 8. The inoculation of rabbits

	INOCULATION (ML)	ROUTE
DAY 0	0,5	Subcutaneous
DAY 3	0,5	Intravenous
DAY 7	1	Intravenous
DAY 10	2	Intravenous
DAY 14	3	Intravenous
DAY 17	3	Intravenous
DAY 21	3	Intravenous

On the 28th day, the rabbits who weighed 3,3 – 3,6 kg were individually bled. The blood was collected and stored at 37 degrees Celsius for one hour, then stored at 4 degrees Celsius until the next day. On the 29th day, the serum was collected from each rabbit and stored at minus 20 degrees Celsius. During the following few weeks, each serum produced was tested with every antigen, made of every strain type. It was then tested with 3-5 of their homologous strains. Serum that performed satisfactorily for a minimum of 6 or 7 dilution was deemed to be fit for purpose. An exception was made for serotype A2 in which 4 dilutions was sufficient. In order to use the sera, it was diluted 10x with a normal saline solution and 50µl merthiolate was added for preservation purposes.

PE/EA/3369-6/2016 – Number of permission of the Department of Epidemiology and Infectious Diseases to perform animal trials.

Preparation of the antigen

1. Washing of the blood

One full Wassermann tube (12ml) of defibrinated sterile sheep blood is centrifuged for 10 minutes, at a speed of 2500 rpm. The supernatant is discarded using a pipette and 5ml of normal saline is pipetted onto the remaining sediment. This process is carried out 3 more times and no saline is added on the final round.

2. Preparation of the antigen

The samples being analysed were biological fluid from either the respiratory tract or spleen of cattle. The strains to be examined were inoculated 24 hours before. One Wassermann tube is prepared for each antigen. Each tube is labelled and 5ml of saline is pipetted into each tube. Using a swab, the bacterial colonies are transferred from the agar gel into the tubes and marked accordingly. The samples are homogenised with a vortex and are placed into a warm water bath at 56 degrees Celsius for 30 minutes. They are allowed to cool to room temperature and 50 microlitres of washed blood is pipetted into each tube. The tubes are placed into a second water bath at 37 degrees Celsius for 60 minutes. After the 60 minutes, the test tubes are taken out of the water bath and the caps are removed to centrifuge the samples. They are centrifuged for ten minutes at 2500 rcf for 3 rounds, ensuring the supernatant is discarded and 5ml of normal saline is added after each round. The test tubes are filled with 10ml and are gently shaken to mix the sediment.

In order to measure the hemagglutination of the RBC and hyperimmune sera, U shaped 96 well plates are used. The rows are labelled accordingly. To every well 50 microliters of saline is added, while 50 microliters of sera is added to the wells of the first column only. Using a pipette, two-fold serial dilution is used to dilute the sera until the penultimate column, as the last column is used as the control. 50 microliters of antigen are pipetted into every well.

The well plates are placed on a white background and are incubated at room temperature for minimum one hour. After incubation, the reaction is observed

macroscopically. If the specific antigen is present in the sample, the RBC will not agglutinate resulting in a sedimentation of the cells. A positive result is indicated by the lack of RBC settling in at least 4 dilutions.

Difficulties often arose whereby a doubtful result was obtained. Frequently, more than one serotype agglutinated at the same time, the control column agglutinated, or the agglutination was seen in less than 4 dilutions. When these instances occurred, the IHA was repeated up to three separate times on separate occasions. If the result remained ambiguous, a conventional PCR test was carried out to identify the bacteria.

Owing to the fact serotype A2 is a weak antigen, this study assigned two rows to A2 with different sera in the U-shaped well plates. It was also accepted as a positive reaction when there was a lack of RBC settling in at least 3 dilutions because of its weak antigenicity. Due to the fact that T3 and T15 show cross reactions during serotyping, the definitive serotype was defined by the serotype that showed the strongest reaction i.e. the highest number of wells in which RBC were settling.

The outcomes of the coagglutination examinations using bacterial suspensions of *Mannheimia* species strains as antigens and coagglutinating agents comprised of hyperimmune sera generated against the type strains of *Mannheimia* are detailed in figure 9. It illustrates the maximal strength of reactions of each serum with each strain and the potential cross reactions.

Figure 9. Sera reaction table

Antigenes	Sera																
	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12	A13	A14	T15	A16	A17
A1	7+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A2	-	3+ / 5+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-
T3	-	-	5+	-	-	+ +/-	-	-	-	-	-	-	-	-	3+ +/-	-	+
T4	-	-	-	5+	-	-	-	-	-	-	-	-	-	-	+/-	-	-
A5	-	-	-	-	3+ +/-	+	- +	-	-	-	-	-	-	-	-	-	-
A6	-- ++ +/-	+/-	-	-	-	8+	-	-	-	-	-	-	-	-	-	-	+/-
A7	-	-	-	-	-	-	6+ +/-	-	-	-	-	-	-	-	-	-	-
A8	-	-	-	-	-	-	-	4+ +/-	-	-	-	-	-	-	-	-	-
A9	-	-	-	-	-	2+	- +	+	7+	-	-	-	-	-	-	-	-
T10	-	-	-	-	-	+	-	-	-	5+	-	-	-	-	+/-	-	-
A11	-	+	-	-	-	+	-	-	-	-	7+	-	-	-	-	-	+
A12	-	-	-	-	-	-	-	-	-	-	-	6+	-	-	-	-	-
A13	-	-	-	-	-	-	-	-	-	-	-	-	6+	-	-	-	-
A14	-	-	-	-	-	-	-	-	-	-	-	-	-	6+	-	-	-
T15	-	-	3+	-	-	+/-	-	-	-	-	-	-	-	-	5+	-	-
A16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6+	-
A17	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	8+

Non typable strains

When non typable strains were found, the IHA test was repeated at least three times per strain. If it remained non-typable, a PCR test was carried out to determine the strain. DNA was extracted using the GBB 100 Presto™ Mini gDNA Bacteria Kit and a conventional PCR method was used based on the real time PCR described by Kumar et al. (2015) and Killion et al. (2018) [41, 61].

A polymerase chain reaction assay targeting *M. haemolytica* serotype-1 specific antigen Rpt2 was used to detect *M. haemolytica*, while LKTBt-1 gene was used. to detect *B. trehalosi*. The primers used are listed in the figure 10.

Figure 10. Genes & primers used for PCR

	Quantity (µl)	***M. haemolytica***	***B. trehalosi***
Primer 1-forward	0,25	Rpt2 GTTTGTAAGATATCCCATTT	LKTBt-1 (name of gene) AGAGGCGAGCGTACTTGTTC
Primer 2-reverse	0,25	CGTTTTCCACTTGCGTGA	ATAGACTCCTCAAGTGGGCTGA

The primers are specific to each species and *M. glucosida* is negative by both primers. The PCR machine used was the Biometra- T personal and the PCR program used is outlined in figure 11.

Figure 11. PCR programme

Step	***M. haemolytica***	***B. trehalosi***
1	95 °C - 3 min	95 °C - 2 min
2	95 °C - 1 min	95 °C - 10 sec
3	48 °C - 1 min	59 °C - 30 sec
4	72 °C - 30 sec	72 °C - 30 sec
Repeating 2-4	34x	39x
5	72 °C – 5 min	72 °C – 5 min

Due to the fact that a conventional PCR was used, the results were obtained using gel-electrophoresis. In the case of positive results, similar sized DNA fragments in the gel and the control strain were noted

Results

A total of 82 samples were tested. Seventy- two were characterised as *M. haemolytica* species, two were characterised as *M. glucosida* and eight as *Bibersteinia trehalosi*. Overall, 41.7% (30/72) of the isolates were identified as serotype A1, 43.1% (31/72) as A2, and 11.1% (8/72) as A6. 2.8% (2/72) were classified as A16 and there was one non typable isolate (1.3%, 1/72). Two isolates represented serotype A11 also known as *M. glucosida*. This data is visually organised in figures 12 and 13.

Regarding *B. trehalosi* 75% (6/8) of the isolates represented T4 while T15 was present as 12.5% (1/8) of the isolates. One strain was non typable (12.5%, 1/8). Figure 14 summarises the B. trehalosi isolates.

The distribution of serotype A1, A2 and A6 were widespread across Hungary. Serotype A11 was only isolated twice from two settlements called Békés and Nyárlörinc, which are both located East of the river Danube. Serotype A16 was isolated from both the West and the East of the river Danube. Serotype T4 was isolated four times from the western aspect of the river Danube and only once from the Eastern part. Two of the western regions in which T4 was isolated, namely Somogytúr and Gecse are in a close proximity to each other. T15 was isolated once and found slightly East of the river Danube in a settlement called Kiskunlacháza.

Serotype A1 was most the most frequently isolated serotype from the lungs of cattle. Serotype A2 which is believed to be a non-pathogenic commensal organism on the upper respiratory tract of bovine animals was not only isolated from the nose and lungs but also the bronchi, blood and spleen. Serotype T4 and T15 were both isolated from the pneumonic lungs of cattle.

Figure 12. Serotypes of *Mannheimia haemolytica* isolated from cattle

	LUNG	NOSE	BAL	SPLEEN	BLOOD	BRONCHI	N/A	TOTAL	%
A1	21	2	4	1	-	-	2	30	41.7
A2	19	8	1	1	1	1	-	31	43.1
A6	4	-	1	-	-	-	3	8	11.1
A16	-	1	1	-	-	-		2	2.8
NT	1	-	-	-	-	-	-	1	1.4

Figure 13. *Mannheimia glucosida* isolated from cattle

	LUNG	NOSE	BAL	SPLEEN	BLOOD	BRONCHI	N/A	TOTAL	%
A11	2	-	-	-	-	-	0	2	100

Figure 14. Serotypes of *Biberstenia trehalosi* isolated from cattle

	LUNG	NOSE	BAL	SPLEEN	BLOOD	BRONCHI	N/A	TOTAL	%
T4	6	-	-	-	-	-	-	6	75
T15	1	-	-	-	-	-	-	1	12.5
NT	1	-	-	-	-	-	-	1	12.5

Discussion

In cases of cattle displaying BRD symptoms, it is possible for them to be infected by more than one causative agent, yet *M. haemolytica* is consistently identified as a contributing factor in BRD [62]. Prior research has primarily concentrated on the occurrence and susceptibility to antimicrobials however, few studies have identified the *M. haemolytica* serotype responsible for respiratory disease in cattle. It is clear that variance exists in different regions of the world.
The findings presented here are in alignment with those of other authors who described the high prevalence of A1 in BRD cases [3, 18, 63]. However, in this study A2 which is frequently reported as being found in healthy animals, represented 43.1% of the *M. haemolytica* samples isolated. This study sheds light on a novel finding: serotype A2 emerging as the predominant strain isolated from pneumonic cattle, which aligns with the result of a thesis [21] where serotype A2 comprised 47.8% of the isolated samples, in contrast to A1, which accounted for only 13%. This is a similar finding to the European study carried out by Andrés-Lasheras et al. (2019) in which serotype A2 strains were also isolated from sickly cattle also [19]. The notable increase of this strain is a considerable increase from Fodor and his colleagues' study in which serotype A2 percentage was 24% [16]. These findings imply that the widely held belief that *M. haemolytica* A2 isolates are predominantly harmless commensal bacteria linked to healthy cattle should be approached with caution.

The third most common serotype isolated in this study is A6. This differs from the Hungarian study undertaken by Fodor in which this serotype was not isolated [16]. An increased incidence of serotype A6 in cattle has been reported in Europe and Japan [18, 20, 22]. A further study undertaken by Katsuda in 2013 revealed that in contrast to the bacteria of other serotypes, those of serotype A6 demonstrated notably elevated rates of antimicrobial resistance, which is of great concern [1].

This study isolated *M. glucosida* on two different occasions from affected lung tissue. No published study indicating this finding has come to my attention however the thesis [21] isolated A11 on one occasion from the lungs. Perhaps

this is attributable to the past inclination of grouping isolates within the *M. haemolytica* complex without differentiating *M. glucosida* from *M. haemolytica* unless specific PCR methods to detect serotype A11 are utilised. This issue underlines the significance of Angen et al. (1999) extensive description of *Mannheimia* and its five species- *M. haemolytica*, *M. glucosida*, *M. granulomatis, M. ruminalis* and *M. varigena*, enabling diagnostic teams to correctly identify distinct taxa [10,11].

During Fodors study of Hungarian farm animals, it is worth noting that the non-typable strains were the majority of strains isolated from extrapulmonary sources, a similar finding noticed by Quirke et al. (1986) however, in this study isolates of *M. haemolytica* were successfully isolated from different organs [16, 24].

Previous serotyping investigations conducted in Hungary revealed solitary isolates of the *B. trehalosi* species [16, 21]. However, this study found seven *B. trehalosi* strains in which 75% of the isolates represented T4 while T15 was present as 12.5% of the isolates. This result is in agreement with the idea supported by Collins (2011) and Cortese et al. (2012) that the isolation of *B. trehalosi* from cattle is increasing, despite the lack of literature on it [32, 33].

A notable feature of this study was the high percentage of isolates that were typable (97.6%) which is higher than previously reported [16]. There were only two non typable strains in this study. This could be attributable to the PCR identification process in which certain strains initially identified as *Mannheimia* species or *B. trehalosi* were subsequently classified as non typable, showing negative results in both PCR tests. Several researchers referenced in Frank's work (1989) have suggested the possibility that strains initially classified as non-typable (NT) through IHA might actually belong to serotypes identifiable through IHA [14]. However, their unresponsiveness to IHA may be attributed to the absence of serotype-specific antigens on their cell surfaces. To ascertain their potential serotype, PCR testing can be employed, particularly when the strain falls within a serotype that can be detected through this method. Alternatively, culturing these strains in a blood-rich environment may facilitate the recovery of

their antigens, although this approach does not consistently yield successful results.

Non-typeable strains may potentially belong to a new serotype, though the probability of identifying a new serotype remains exceedingly low, primarily due to the fact that the last discovery of a new *Mannheimia haemolytica* serotype occurred decades ago. To explore the possibility of uncovering fresh serotypes, the production of new serum in rabbits is a prerequisite. However, it is advisable to first determine the serotype of non-typeable strains using a PCR method. This preliminary step is recommended since the serum production process is both time-consuming and financially demanding.

Vaccines are available; however, their effectiveness remains questionable. This can be owed to the fact that the surface antigens of *M. haemolytica* differ among serotypes, making cross protection very challenging. A limited number of studies examine this cross protection and only one commercial vaccine claims to exhibit cross protection [53]. However, this was exhibited under laboratory conditions further underling the need of undertaking future experiments under field conditions rather than using experimental challenges, thereby allowing the natural exposure of pathogens [59]. All vaccines commercially available only target one *M. haemolytica* serotype- A1. This study found A2 to be the most predominately isolated serotype followed by serotype A1 and A6 in which A2 is not targeted and only one vaccine claims to exhibit cross protection with serotype A6. No vaccine targets serotype A11 or A16, which were also isolated in this study. No vaccine exists to target *B. trehalosi* serotypes although cross protection has been described between *M. haemolytica* and *B. trehalosi* [33, 58].

It is clear that one of the primary methods to decrease the emergence of BRD is the vaccination of cattle against the main pathogens especially *M. haemolytica* and *B. trehalosi* [64]. It is disheartening that such a large gap in our knowledge exists regarding this essential tool in our fight against BRD. Nevertheless, the identification of the specific serotypes prevalent in certain regions stands as a critical phase in the management of BRD, as it provides essential information in the production of vaccines.

Conclusion

The findings of this study offer valuable insights into the serotype distribution of *M. haemolytica* and *B. trehalosi* in Hungary. This study demonstrates that although serotype A1 of *M. haemolytica* continues to play a major role in the pathogenesis of BRD, the role of serotype A2 should be investigated further. Veterinarians should maintain a high level of vigilance when monitoring *B. trehalosi*, as it is increasingly becoming a prominent bacterial pathogen within the BRDC complex. Furthermore, this study brings to the forefront the limitation of current commercial vaccines, which may contain only *M. haemolytica* serotype A1, and provide serotype specific protection, and thus do not offer comprehensive protection against *M. haemolytica* and *B. trehalosi*. BRDC continues to be a challenging health issue facing cattle worldwide and surveillance programs for serotypes play a crucial role in ensuring that vaccines under development encompass all serotypes associated with pathogenicity.

Abstract

Bovine respiratory disease complex (BRDC) is one of the most common and costly disease in cattle production worldwide. The multifactorial nature of BRDC necessitates comprehensive research and targeted strategies for prevention, including identification and serotyping of prevalent strains and vaccination combined with improved management practices, to mitigate its impact on cattle health and productivity. The last major serological examination of *Mannheimia haemolytica* and *Bibersteinia trehalosi* in Hungarian cattle was published twenty-four years ago, highlighting the timeliness and relevance of undertaking this research again.

Over a period of six years (2017-2023), a total of 72 *M. haemolytica 2 M. glucosida* and 8 *B. trehalosi* strains were isolated from cattle presenting with clinical signs of BRDC in Hungary. The isolates were serotyped using passive hemagglutination. The identification of the strains on species level was confirmed by PCR.

With regards to *M. haemolytica* this study identified a predominance of serotypes A2 (43.1%, 31/72), A1 (41.7%, 30/72), and A6 (11.1%, 8/72). 2.8% (2/72) of the isolates were classified as A16 and there was one non typable isolate (1.3%, 1/72). Two isolates represented serotype A11 also known as *M. Glucosida*.
With regards to *Bibersteinia trehalosi*, 75% (6/8) of the isolates represented T4 while T15 was present as 12.5% (1/8) of the isolates. One strain was non typable (12.5%, 1/8).

The findings of this study offer valuable insights into the prevalence of serotypes associated with pneumonic pasteurellosis in Hungarian cattle. Based on these findings, contrary to previous beliefs, but in accordance with the previous Hungarian data, *M. haemolytica* serotype A2 and *B. trehalosi* serotype T4 may be considered as primary pathogens of respiratory disease in cattle and should be investigated further. Furthermore, this study brings to the forefront the limitation of current commercial vaccines, which mainly contain only the *M.*

haemolytica serotype A1, and provide serotype-specific protection, therefore, do not offer comprehensive protection against *M. haemolytica* and *B. trehalosi*. A comprehensive understanding of the prevalence of these organisms and their serotypes informs a focused strategy for the manufacture of vaccines against BRDC.

Absztrakt

A szarvasmarhák légzőszervi komplex betegsége (BRDC) a szarvasmarhatartásban világszerte az egyik leggyakrabban előforduló és legnagyobb gazdasági károkat okozó betegség. A BRDC multifaktoriális természete átfogó kutatást és célzott megelőzési stratégiát tesz szükségessé, mely magában foglalja az előforduló törzsek azonosítását és szerotipizálását, a vakcinázást és a megfelelő tartáskörülményeket. Így enyhíthetjük a betegség káros állategészségügyi hatásait és termeléscsökkenést. A magyarországi szarvasmarhákban előforduló *Mannheimia haemolytica* és *Bibersteinia trehalosi* törzsek legutóbbi nagyobb átfogó szerológiai vizsgálatát 24 éve publikálták, így időszerűvé vált a téma újbóli vizsgálata.

Összesen 72 *M. haemolytica, 2 M. glucosida* és 8 *B. trehalosi* törzset vizsgáltunk, melyeket a legutóbbi 6 év (2017-2023) során, BRDC klinikai tüneteit mutató, magyarországi szarvasmarha -állományokból izoláltak. A törzseket passzív hemagglutinációs módszerrel szerotipizáltuk. A törzsek faj szintű határozását PCR-rel is megerősítettük.

A munkánk során az A2 (43.1%, 31/72) az A1 (41.7%, 30/72) és az A6 (11.1%, 8/72) szerotípusok bizonyultak a leggyakoribbaknak. 2-2 törzset (2,8%) A16-os és A11-es szerotípusúként határoztuk meg, mely utóbbi a *Mannheimia glucosida* fajt jelenti. A *B. trehalosi* tekintetében a T4-es szerotípus dominált, a törzsek 75%-át tette ki(6/8), míg a T15-ös szerotípust 1 esetben (12,5%) észleltük. Egy-egy *M. haemolytica* (1.3%, 1/72) és *B. trehalosi* 12.5%, 1/8) nem besorolható szerotípusú volt.

A kutatásunk bepillantást enged a magyarországi szarvasmarhákban légzőszervi pasteurellosist okozó törzsek szerotípusainak megoszlására. Korábbi megállapításokkal ellentétben, de az előző magyarországi adatokkal összhangban, a *M. haemolytica* A2-es és a *B. trehalosi* T4-es szerotípusai elsődleges kórokozóknak tekintendők szarvasmarhák légzőszervi betegségei esetében és további vizsgálatra érdemesek. Ez a tény rávilágít a jelenleg elérhető vakcinák korlátjaira: mivel a védelem szerotípus függő és a vakcinák többsége csak a *M. haemolytica* A1-es szerotípusát tartalmazza, így nem biztosítanak teljes körű védelmet. A vizsgált baktériumok szerotípusainak átfogó vizsgálata és megértése segíti a BRDC ellen használt vakcinák fejlesztését.

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