Eurasian Journal
of Veterinary Sciences

www.eurasianjvetsci.org

RESEARCH ARTICLE

The enigmatical manipulators in the capsule synthesis of *Pasteurella multocida*: Iron acquisition proteins

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Received: 13. 04.2023, Accepted: 23.08.2023

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Pasteurella multocida'nın kapsül sentezindeki esrarengiz manipülatörler: Demir alım proteinleri

Eurasian J Vet Sci, 2023, 39, 3, 139-143
DOI: 10.15312/EurasianJVetSci.2023.409

Öz

Amaç: *Pasteurella multocida*'daki spontan kapsül kaybı veya kapsül değişiklikleri, tekrarlanan laboratuvar geçişlerinden, pozitif veya negatif düzenleyici genlerden veya bilinmeyen bir genden kaynaklanabilir. Bu çalışmada, tipik olmayan ve tipik *P. multocida* suşlarının fenotipik, genotipik ve biyotipik özelliklerinin karşılaştırılması, kapsül sentezindeki baskın genlerin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Bu çalışmada kapsül tipi belirlenen 56 suş ve kapsül tipi belirlenemeyen otuz altı suş kullanıldı. İzolatlarda baskın genlerin (serogrup, serotip, toksin, adezin, demir alımı ve koruyucu) varlığına dayalı olarak çoklu doğrusal regresyon analizi kullanıldı.

Bulgular: Bu suşların kültür yöntemleri ile koloni morfolojileri değerlendirildiğinde, tipik suşlarda (%87,5) mukoid koloni oluşumu, tipik olmayan suşların aksine (%27,7) yaygın olarak saptanmıştır. Tipik suşlarda en yüksek ptfA, ompA ve tadD gen yüzdeleri sırasıyla %78,57, %75 ve %69,64 idi. Tipik olmayan suşlarda en yüksek ompA, ptfA ve tadD gen oranları sırasıyla %61,1, %52,78 ve %52,78 idi. Çoklu lineer regresyon analizi sonuçlarına göre, hgbA ve hgbB genlerinin birlikteliği tipik olmayan suşlarda kapsül sentezinin artmasına neden olmuştur. Bu suşlarda ompA geninin varlığı, ikinci olarak bir indüksiyondu. Diğer genler, tipik olmayan suşlarda kapsül sentezinde etkili değildi.

Öneri: Tipik olmayan *P. multocida* suşlarının oluşumundaki en önemli etkinin HgbA ve HgbB genlerinin yeterli olmaması ile ilgili olduğu belirlendi. P. multocida'nın demir kısıtlamalı koşullar altında yoğun bir şekilde kapsüllenmemiş olabileceği düşünüldü. Sonuç olarak, *P. multocida*, demir alma proteinlerine bağlı olarak kapsülünü değiştirebilir veya kapsülünü kaybedebilir.

Anahtar kelimeler: Çoklu linear regresyon analizi, *Pasteurella multocida,* pnömoni, virülans ilişkili genler

Abstract

Aim: Spontaneous capsular loss or capsular changes in *Pasteurella multocida* can result from repeated laboratory passages, positive or negative regulatory genes, or an unknown gene. This study, it was aimed to compare the properties of phenotypic, genotypic, and biotypic of each non-typical, and typical *Pasteurella multocida* strain, to determine the dominant genes on capsule synthesis.

Materials and Methods: Fifty-six strains, which capsular type was determined, and thirty -six, which capsular type was not determined, were used in this study. Multiple linear regression analysis was used based on the presence of dominant genes (serogroup, serotype, toxin, adhesin, iron acquisition, and protectin) in the isolates.

Results: When colony morphologies of strains were evaluated of these strains by culture methods, mucoid colony formation was commonly detected in typical strains (87.5%), in contrast to non-typical strains (27.7%). In typical strains, the highest percentages of ptfA, ompA, and tadD genes were 78.57%, 75%, and 69.64%, respectively. In non-typical strains, the highest rates of ompA, ptfA, and tadD genes were 61.1%, 52.78%, and 52.78%, respectively. According to multiple linear regression analysis results, the together hgbA with hgbB genes caused the increase of capsule synthesis in these strains. The presence of the ompA gene in these strains was secondly a induction on these strains. Other genes were not effective in capsule synthesis in these strains.

Conclusion: It was determined that the most significant effect in the forming of non-typical *P. multocida* strains was related to not enough HgbA and HgbB genes. It was supposed that *P. multocida* may not be heavily encapsulated under iron-restricted conditions. Consequently, *P. multocida* may change its capsule or lose its capsule related to iron acquisition proteins.

Keywords: *Pasteurella multocida*, pneumonia, multiple linear regression analysis, virulence-associated genes

CITE THIS ARTICLE: *Balevi et al., 2023. The enigmatical manipulators in the capsule synthesis of Pasteurella multocida: Iron acquisition proteins Eurasian J Vet Sci, 39, 3, 139-143*

Introduction

Healthy cattle have pathobionts in their nasal cavities, which are commonly *Pasteurellaceae family*: *Pasteurella multocida, Histophilus somni,* and *Mannheimia haemolytica* (Capitini et al 2002). *Pasteurella multocida* is a heterogeneous species that cause various infection such as avian fowl cholera, snuffles in rabbits, enzootic pneumonia, and bovine hemorrhagic septicemia in a wide range of animal species (Weber et al 1984). *P. multocida* strains are classified into serogroups based on five capsule antigens (A, B, D, E, and F) and typed primarily on lipopolysaccharide antigens into 16 serotype (1–16) (Carter 1952, Heddleston et al 1972). Also there are the various virulence proteins (adhesins, hyaluronidase, outer membrane and porin proteins, iron acquisition proteins, sialidases, and toxins) (Boyce and Adler, 2000). These factors play important role in the efficiency of vaccination (Ujvari et al 2019). To survive bacteria, iron is an essential element, and it is believed that iron acquisition proteins play a role in the disease process. Through hemin, hemoglobin binding protein (hgb) A and hgbB help for the growth of bacteria (Rimler 2001). Outer membrane protein (omp) A, which is the immunogenic and protected structure of the outer membrane, plays of role in epidemics (Luo et al 1997). OmpA has been used to assess the pathogen's interaction with the host, as well as the association of this construct with infection and its diversity within different species (Lin et al 2002). Since OmpA plays an important role between the host and the pathogen, has high immunogenicity, and has no similarity with other structural proteins of the bacteria, it must be among the components of the vaccine for the vaccine to be effective (Marandi and Mittal 1997).

Especially, particularly serotype A:3 or A:1, can give rise to critical respiratory diseases in cattle and has largely caused epidemics in beef calves (Ewers et al 2006). It is known that acapsular strains (non-typical) of *P. multocida* are less virulent than capsular strains (typical) (Oh et al. 2019). But it has been presented that the isolation rates of non-typical *P. multocida* strains may be different from ~ 0.5% to 10.6% in farm animals with respiratory system infections in the different regions (Harper et al 2006, Riley et al 2020, Shayegh et al. 2008). Interestingly, we detected non-typical *P. multocida* strains with 39.13% from sheep, goats, and calves with respiratory tract infections (Sakmanoglu et al. 2021). Protective immunity was obtained in chickens vaccinated with high doses of acapsular mutant (Chung et al 2003). Because of capsular type variety, a wide range of hosts, and especially acquired immunity of serotype-specific, there are enormous difficulties in the protection with vaccines from this infection (Harper et al 2006).

Therefore, several commercial vaccines do not provide protection from this convention as they do not have the desired level of efficacy (Chung et al 2003).

In this study, it was aimed to compare the fenotypic, genotypic and biotypic properties of each one of non-typical *P. multocida* with typical *P. multocida*, to determine of the dominant genes on capsule sythesis.

Material and Methods

Bacterial strains and culture

A fifty-six strains, which capsular type were determined, and thirty -six, which capsular type were not determined, were used in this study. Also, *P. multocida* type strains (ATCC 12945, NCTC10323, ATCC 12948, and ATCC 43020) were used as positive controls. At least one of the clinical symptoms of respiratory infection as fever, nasal discharge, and cough were seen in all the animals. *P. multocida* was isolated from a blood agar base supplemented with 5% sheep blood, and incubated in a 7% CO2 atmosphere for 24 h at 37°C in. Colony formation results of these strains were investigated as stated in this study (Sakmanoglu et al 2021).

Determination of virulence factors by PCR

This section was carried out in our study previously (Sakmanoğlu et al 2021). Briefly, the Wizard® Genomic DNA Purification Kit (Promega, USA) was used to obtain all DNA extracts from the isolates. The serogroup (capA, capB, capD, capE, capF) (Townsend et al 1998, Townsend et al. 2001, Sakmanoglu et al 2021), and serotype (L1-8, L3A-L6A) (Harper et al 2015) of isolates were determined. Also, iron acquisition (tbpA, hgbA, hgbB, tonB), protectin (ompA, ompH, omp87, plpB), toxin (toxA), and adhesion (ptfA, pfhA, tadD) genes of isolates were determined as described previously protocol (Ewers et al 2006, Sakmanoglu et al 2021).

Statistical analysis

The obtained results were evaluted by multiple linear regression analysis (IBM SPSS Statistic 21 Program) were used to compare the risk values of related genes in the both typical and non-typical strains. These values were p value, spesivite, sensivite, odds ratio, and confidence interval.

Results

When colony morphologies of strains were evaluated of these strains by culture methods, mucoid colony formation was commonly detected in typical strains (87.5%), in contrast to non-typical strains (27.7%). The A capsular type was the most common serogroup in typical (85.71%). The L3A was the most common serotype in typical (69.64%) and non-typical (47.22%) strains. All strains possessed at least one gene from adhesins (tadD, ptfA), toxin (toxA), protectins (plpB, ompH, ompA), iron acquisition (tonB, exbB, hgbA, exbD) in contrast to pfhA with Oma87. In typical strains,

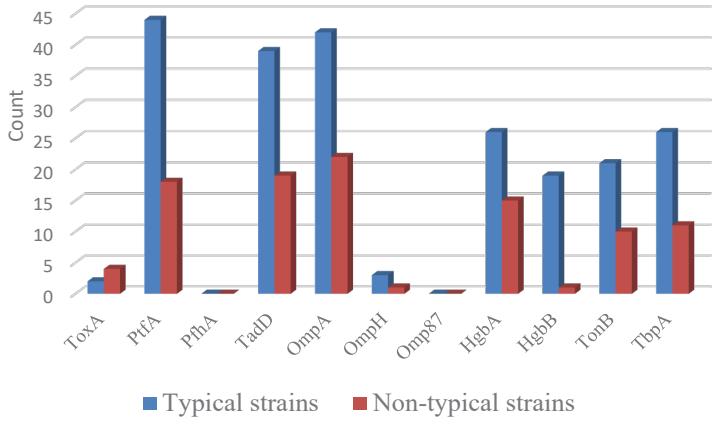

Figure 1. Graphic of virulence-associated genes in both typical and atypical Pasteurella multocida strains

Table 1. Percentages of virulence-associated genes in typical and non-typical Pasteurella multocida strains

Strain types→		Typical strains		Non-typical strains	
Virulence-associated genes↓		Count	%	Count	%
Toxin	ToxA	2	3.57	4	11.11
Adhesins	PtfA	44	78.57	18	52.78
	PfhA	0	0	0	0
	TadD	39	69.64	19	52.78
Protectins	OmpA	42	75	22	61.11
	OmpH	3	5.35	1	2.77
	Omp87	0	0	0	0
Iron acquisition	HgbA	26	46.42	15	41.66
	HgbB	19	33.92	1	2.77
	TonB	21	37.5	10	27.77
	TbpA	26	46.42	11	30.55

the highest percentages of ptfA, ompA, and tadD genes were 78.57%, 75%, and 69.64%, respectively. In non-typical strains, the highest rates of ompA, ptfA, and tadD genes were 61.1%, 52.78%, and 52.78%, respectively (Sakmanoglu et al. 2021) (Table 1, Figure 1). Because, it was found that spesivite, sensivite, and p values of hgbA with hgbB genes were detected 78%, 58%, value <0.05, respectively. Odds ratios of hgbA with 2.933, and hgbB with 32.154 were highest values in the genes. Also, whereas confidence interval up values of hgbA and hgbB were 7.471, and 269.102, respectively, confidence interval low values of hgbA and hgbB were 1.152, and 3.842, respectively. According to regression analysis results, the hgbA with hgbB genes were the highest risk on capsule synthesis in these strains. Presence of ompA gene in these strains were secondly as a possible risk on these strains. Other genes were not effective on capsule synthesis in these strains.

Discussion

Capsule (A, D, and F) structure, composed of chondroitin, hyaluronic acid (HA), and heparin is known, better than structures of serogroup E and B capsules, which have a more complex structure (Cifonelli et al 1970, DeAngelis et al 2002). Capsular type A of *P. multocida* causes respiratory disease in cattle (Ewers et al 2006). Mucoid colony of *P. multocida* strain is observed in lung samples of cattle, rabbits, and pigs although non-mucoid colonies are isolated from poultry (Harper et al 2006, Gluecks et al 2017).

Previous to our study, we detected that the rate of non-typical strains was interestingly more than that reported so far, at the same time we isolated from farm animals with respiratory disease. Also, mucoid colony formation was commonly detected in typical strains (87.5%), in contrast to non-typical strains (27.7%) (Sakmanoglu et al 2021). This variation has been ignored until now. Because of various determinants, spontaneous capsule loss has been seen in *P. multocida* (Steen et al 2010, Smallman et al 2022).

Capsule spontaneous loss in *P. multocida* can originate from the repeated passage of one then more (30 sub-cultures) (Muniandy et al 1992, Steen et al 2010). According to sequence analysis results, these acapsular variants are caused by two nucleotide changes in the cap locus, but these changes were not explained in how being on effective the acapsular phenotype formation (Watt et al 2003). Factor for inversion stimulation (fis) (Steen et al 2010) with hfq (Smallman et al 2022) genes encode known positive regulators of *P. multocida* capsule. There need for information about the cellular signals, which control regulatory mechanisms and capsule production in *P. multocida*. It was reported that the regulator fis both controls the expression of capsule biosynthesis genes and regulates known and putative

virulence factors in *P. multocida* (Dorman et al 2018). Also, fis proteins are synthesized at the highest level in the active growth phase of bacterial cells contrary to the stationary phase (Steen et al 2010). Iron plays a critical role in metabolic electron transport chains for most organisms. Transferrin and lactoferrin in body fluids in avian and mammalian hosts can affect the concentration of free iron normally present and the growth of bacteria in vivo impress with negative because of less iron amount (Bullen 1981). Therefore, to survive negative conditions, pathogens must possess an effective response to protect from the limited iron conditions encountered upon entry into a host (Veken et al 1996). It is reported that iron acquisition proteins play a role in the disease process, because hemoglobin binding protein (hgb) A and hgbB help for the growth of bacteria (Rimler 2001). The hgbA and HgbB proteins are used to obtain iron directly from the haem component. The prevalence of hgbB gene in strains alters relative to the host origin and the animal disease status, while hgbA gene is more regularly among isolates. TbpA, an epidemiological marker among cattle, plays an essential in the obtaining of iron from transferrin by transferrin-binding protein role (Paustian et al 2001). Iron-restricted conditions with iron deprivation effects markedly in decreasing the capsular amount of *P multocida*. These chelators affecting capsule structure are inhibited by the addition of iron neutralized (Jacques et al 1994).

Conclusion

In conclusion, it was determined that the most significant effect on the capsule synthesis of *P. multocida* was related to HgbA and HgbB genes. *P. multocida* may not be heavily encapsulated under iron-restricted conditions. Additionally, *P. multocida* may change its capsule or lose its capsule related to iron acquisition proteins.

Acknowledgements

A part of this study was presented at the 1st International Congress of Selcuk Health Sciences (2022).

Conflict of Interest

The authors did not report any conflict of interest or financial support.

Funding

This research was a section of a project financed by the Coordinatorship for Scientific Research Projects of Selcuk University, Konya, Turkey [grant number 19401156].

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Author Contributions

Motivation/Concept: AB; Design: ZS; Control/Supervision: OE; Data Collection and/or Processing: AI, AU; Analysis and/or Interpretation: BP; Literature Review: AG; ET; Writing the Article: AB; Critical Review: AB, AI

Ethical Approval

This research has been approved (grant number: 2020-69, Date: 20.08.2020) by the Ethics Committee of the Faculty of Veterinary Medicine at the University of Selcuk in Konya, Turkey.