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Pasteurella multocida effectively utilizes hyaluronic acid to facilitate its continuous growth - short comminication.

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ABSTRACT

Pasteurella multocida B: 2 (*P. multocida*) causes haemorrhagic septicemia (HS), a fatal disease in cattle and buffalo. Despite its widespread prevalence, little is known about the factors that contribute to *P. multocida's* pathogenicity. This pathogen produces hyaluronidase, a hyaluronic acid degrading enzyme. However, its role in *P. multocida* pathogenicity is unknown. In this study we attempted to assess the potential of *P. multocida* in utilising hyaluronic acid as a nutrient *in vitro*. Six isolates of *P. multocida,* isolated from outbreaks of HS, were examined for their growth in a chemically defined medium (CDM) with glucose, and a CDM without glucose but with hyaluronic acid (HA) added. The bacterial growth was determined by counting the number of colonies at each observation time (24, 48, 72, 96 h), and was expressed as CFU millions/mL $\log_{10}$. *P. multocida* continued to grow throughout the period of the experiment in the CDM with HA. However, in the CDM with glucose, growth could be observed until 72 h, followed by a decline and then no growth after that. This is the first ever report of *P. multocida* utilising hyaluronic acid for its growth, a strategy that could be used to obtain nutrients for colonisation and proliferation.

Key words: *P. multocida*; Hyaluronidase; hyaluronic acid; CDM

Introduction

P. multocida is a common commensal and opportunistic pathogen that resides in the upper respiratory tract of healthy animals. It has been associated with a variety of respiratory illnesses in various host species (WILKIE et al., 2012). In cattle and buffalo, it produces heamorrhagic septicemia (HS), an acute, deadly septicemic disease resulting in major morbidity and mortality, as well as large economic losses, mainly in Asian and African countries (KUTZER et al., 2021). HS disease appears to be more severe in buffalo, in terms of susceptibility, morbidity and fatality, when compared to cattle. It has emerged as a major infectious disease in India causing heavy economic

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loss, much of which is the result of India's large population of cattle and buffaloes (SINGH et al., 2014).

HS is classified as a List B disease by the Office International des Epizooties (OIE) and is caused by two serotypes of *P. multocida:* B:2 (Asian serotype) and E:2 (European serotype) (African serotype). In the absence of treatment, the case fatality may reach 100 per cent (BENKIRANE and DE ALWIS, 2002). The disease is so fatal that in an outbreak of HS disease in central Kazakhstan, over 200,000 saiga antelopes died in three weeks, leading to a catastrophic drop in saiga populations and driving them to the edge of extinction (KOCK et al., 2018).

The capsule, lipopolysaccharides, various adhesions, iron acquisition protein, outer membrane proteins (OMP), and the extracellular enzymes neuraminidase (sialidase) and hyaluronidase have all been identified as virulence factors of *P. multocida* (EWERS et al., 2006).

The enzyme hyaluronidase has been identified as a virulence factor in a variety of Gram positive and Gram-negative bacteria (HYNES and WALTON, 2000; HAAS et al., 2015). This enzyme is reported to facilitate the spread of bacteria and toxins, and thus play an important role in disease pathogenesis (STARR and ENGLEBERG, 2006). In (1980), CARTER and CHENGAPPA discovered that *P. multocida* produces hyaluronidase. Interestingly it was produced specifically by serotype B:2, which causes HS in cattle and buffaloes. Hyaluronidase is an enzyme that degrades hyaluronic acid (HA), which is a major component of animal tissue's extracellular matrix, and is composed of repeating disaccharide units of β-1,4-D-glucuronic acid-β-1,3-*N*-acetyl-β-D-glucosamine by β-elimination at the β-1,4 glycosidic linkages leading to Δ 4,5-unsaturated oligosaccharides. Hyaluronic acid is widely distributed in connective tissues such as the umbilical cord, synovial fluid and skin, as well as existing in substantial amounts in the lungs, brain, kidney, and muscle. In the respiratory system, it is found in the normal respiratory submucosa, the pulmonary blood vessel wall, and to a lesser extent in the alveoli (LAUER et al., 2015).

Pathogenic bacteria have been reported to produce hyaluronidase enzymes for HA degradation, which promotes adhesion, colonisation, and the spread of the organism in host tissues (HYNES and WALTON, 2000). The enzymatic degradation of hyaluronic acid has been associated with wound infections, pneumonia, bacteremia, septicemia, bovine mastitis, gas gangrene, meningitis, and toxic shock syndrome, among other clinical outcomes (MATSUSHITA and OKABE, 2001; JEDRZEJAS, 2004; MAKRIS et al., 2004; WU et al., 2010; SCHMIDT et al., 2012; CHELAZZI, et al., 2015). As a result, hyaluronidase is regarded as a potential virulence factor of pathogenic bacteria.

Some studies suggest that the bacteria that produce hyaluronidase may use HA as a nutrient. Staphylococci and Streptococci strains implicated in infectious endophthalmitis were studied in an experiment to see if they could use hyaluronic acid as a nutrient. They discovered that the bacteria could grow in a hyaluronic acid-containing medium by using it as a nutrient (COSTAGLIOLA et al., 1996). In a similar experiment, *Mycobacterium tuberculosis* was found to use hyaluronic acid as a carbon source (HIRAYAMA et al., 2009). In another experiment, *Streptococcus pneumoniae*, the causative agent of bacterial meningitis and septicaemia, was shown to use hyaluronic acid for growth in an otherwise low carbohydrate region of the respiratory airways (MARION et al., 2012; YADAV et al., 2013).

Despite the fact that *P. multocida* B:2 produces hyaluronidase, it is unknown what role it plays in the pathogenesis of haemorrhagic septicaemia. The goal of this study was to demonstrate the ability of hyaluronidase produced by *P. multocida* to degrade hyaluronic acid, which can be used to promote its growth.

Materials and methods

Bacterial strains, culture media and chemicals. *P. multocida* P 52 B2 serotype, and 6 isolates from outbreaks, were included in the study. The freezed glycerol broth of *P. multocida* P52 B2 serotype was obtained from the Department of Veterinary Microbiology, GADVASU, Ludhiana. The *P. multocida* P52 B: 2 serotype and the isolates were confirmed by a specific polymerase chain reaction (PCR) method (TOWNSEND et al., 1998). The

culture was revived by inoculating it on brain heart infusion (BHI, Hi-Media) agar containing 5% sheep blood, and was grown at 37°C for 12 h. A loopful of one colony of bacteria was added to BHI broth and incubated overnight. The culture broth was kept at 4°C and was used within 12 h.

Assay for Detecting Hyaluronidase-Producing Colonies. Detection of hyaluronidase production by field isolates of *P. multocida* was performed as per HART et al. (2009) with minor modifications.

Preparation of tryptose soy agar hyaluronic acid plates (TSHA) media plates. Six grams of tryptic soy broth were added to 120 ml of distilled water, followed by the addition of 1.2 g of agarose [1% (w/v)]. The solution was autoclaved and kept in a water bath at 50^0C. In a separate beaker, 2 g of bovine serum albumin (BSA; Fraction V; TC-194, HiMedia) [5% (w/v)] was added to 40 ml of autoclaved distilled water. In another beaker, 40 mg of hyaluronic acid (53747, Sigma) was added to 40 ml of autoclaved distilled water (1 mg/mL). The hyaluronic acid was dissolved by rigorous vortexing and heating below 50°C. The BSA solution was then added to the HA solution and kept for 30 min in a water bath at 50°C for equilibration. The BSA HA solution was then finally added to the tryptic soy broth and agarose media. The suspension was mixed and dispensed into Petri plates. The plates were kept overnight in an incubator at 37°C to check for the presence of any contamination. Finally, the media plates were stored at 4°C for a maximum of three weeks.

Preparation of inoculum and detection of hyaluronidase production. A loopful of one colony of bacteria was mixed in 50 µl of autoclaved normal saline. One microlitre of the suspension was then added in a spot manner to the TSHA media plates. The plates were incubated at 37°C for an overnight period (15–18 h). Following incubation each plate was flooded with 2M acetic acid and left to react for 2-5min. Degradation of the hyaluronic acid was detected as a zone of clearing resulting from acetic acid precipitation of undigested HA conjugated to BSA.

Growth Assay. The chemically defined medium was prepared as previously described by JABLONSKI et al. (1996) with and without the addition of carbohydrates. The medium without glucose was supplemented with hyaluronic acid at 3mg/ml (53747, Sigma). A loopful of one colony of bacteria was added to 10 ml of BHI broth in a tube and incubated overnight. The bacteria were then washed and resuspended in 10 ml of PBS, at pH 7.2. Fifty microlitres of washed bacteria were added to 1 ml of the medium with glucose and to the medium without a carbohydrate but containing hyaluronic acid. The tubes were incubated at 37°C for different time periods of 24 h, 48 h, 72 h and 96 h. Following incubation, the bacteria were diluted tenfold and spread in a tilted manner on a BHI agar plate. The plate was incubated overnight at 37°C and colony forming units were counted.

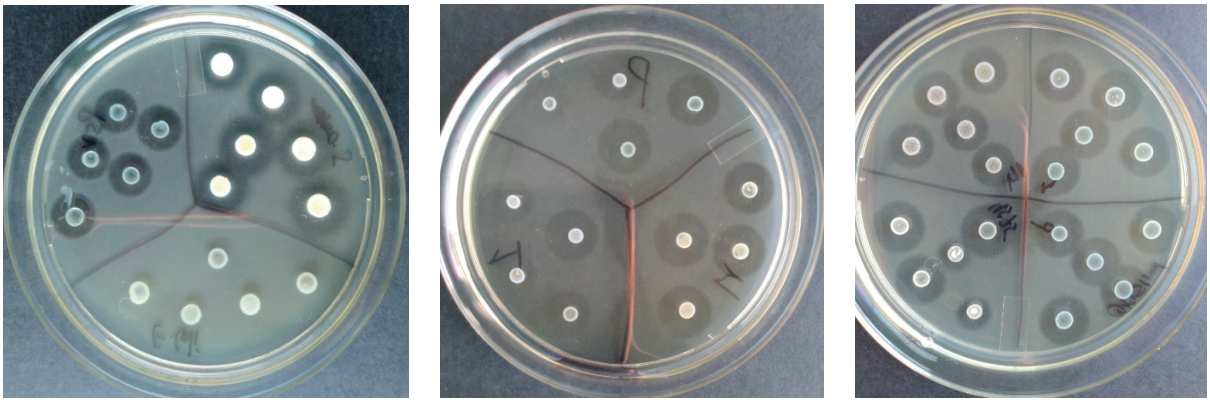
Data Analysis. All data are presented as the means ± standard deviation of the means. Two-way ANOVA with a multiple comparison test on GraphPad Prism was used to compare the growth assay of isolates, presented as CFU (millions/ml) $\log_{10}$) at different incubation periods. A statistically significant value was defined as one with a P-value less than 0.05.

Results

P. multocida can grow at different pH ranging from 6.0-7.2. The chemically defined medium for *P. multocida* was prepared as per JABLONSKI et al., (1996) and used as the growth medium. However, calcium pantothenate in the recommended concentration precipitated when the pH was raised to 7.2. When the pH was maintained at 6.8, the solution was transparent. Thus, we first examined the growth of *P. multocida* in BHI at various pH levels ranging from 5.5 to 7.2, and observed that the growth of *P. multocida* was optimal even at acidic pH levels. As a result, we employed CDM with a pH of 6.6 for the growth assay.

Hyaluronidase production by P. multocida B:2 field isolates. One microlitre of the suspension of *P. multocida* B: 2 field isolates, *E. coli* (negative control) and *S. aureus* (positive control) was added in a spot manner onto the TSHA media plates and incubated at 37°C for an overnight period (15–18 h). The experiment demonstrated a clear halo around the *P. multocida* P52 strain, *Staphylococcus aureus* (positive control) and all 6 field isolates

of *P. multocida* B:2 (Fig. 1). In contrast, *E. coli,* which served as a negative control, did not produce hyaluronidase and hence no clear zone around colonies.



a. P. multocida (P52 vaccine strain, S. aureues (Positive control, E. coli (negative control)

b. P. multocida isolates

c. P. multocida isolates

Fig. 1. Colonies *P. multocida* isolates producing hyaluronidase

a. P. multocida P52(vaccine strain, upper left sector), *Staphylococcus aureus* (positive control, upper right sector) and *E. coli* (negative control, bottom sector) was laid in a spot manner onto the Tryptose soy agar hyaluronic acid plates (TSHA) media plates and incubated for 15-18 h. The clear halo around the colonies demonstrates digestion of hyaluronic acid by the action of hyaluronidase produced by the bacteria. b & c Demonstration of hyaluronidase production by field isolates of *P. multocida.*

P. multocida utilises hyaluronic acid as a nutrient and for sustained growth. According to the data presented above, both the reference strain and field isolates of *P. multocida* produced hyaluronidase. As a result of the speculation that hyaluronidase may interact with hyaluronic acid during infection, we investigated the effect of hyaluronic acid on *P. multocida* growth.

P. multocida field isolates were grown in CDM with glucose and CDM without glucose but hyaluronic acid added, and were incubated at 37°C for 24, 48, 72 and 96 h. The mean ± SE of CFU millions/mL Log_{10} is presented in Table 1 and Fig. 2. The experiment of growth of *P. multocida* in CDM with hyaluronic acid as the only carbon source demonstrated that *P. multocida* was able to grow significantly as compared to with the addition of glucose. The growth of *P. multocida* in CDM with glucose was higher until 48 h, but then declined by 72 h and finally there was no growth at 96 h (Fig. 2-3). On the other hand, *P. multocida* growth in CDM + HA was initially higher, though less than with glucose, but then a significant decrease in CFU was observed by 48 h. However, significant sustained higher growth was demonstrated from 72 h to 96 h.

The lefthand panel of figures a., b., c., d. shows the growth of *P. multocida* in CDM (pH 6.6) with glucose (G) at the incubation periods of 24 h, 48 h, 72 h and 96 h, respectively, with a similar incubation temperature of 37°C. The righthand panel of figures e., f., g., h. demonstrates the growth of *P. multocida* in CDM (pH 6.6) with hyaluronic acid (HA) but without glucose at similar incubation periods and temperature as the growth of *P. multocida* in CDM with glucose. Both experiments were carried out in tandem.

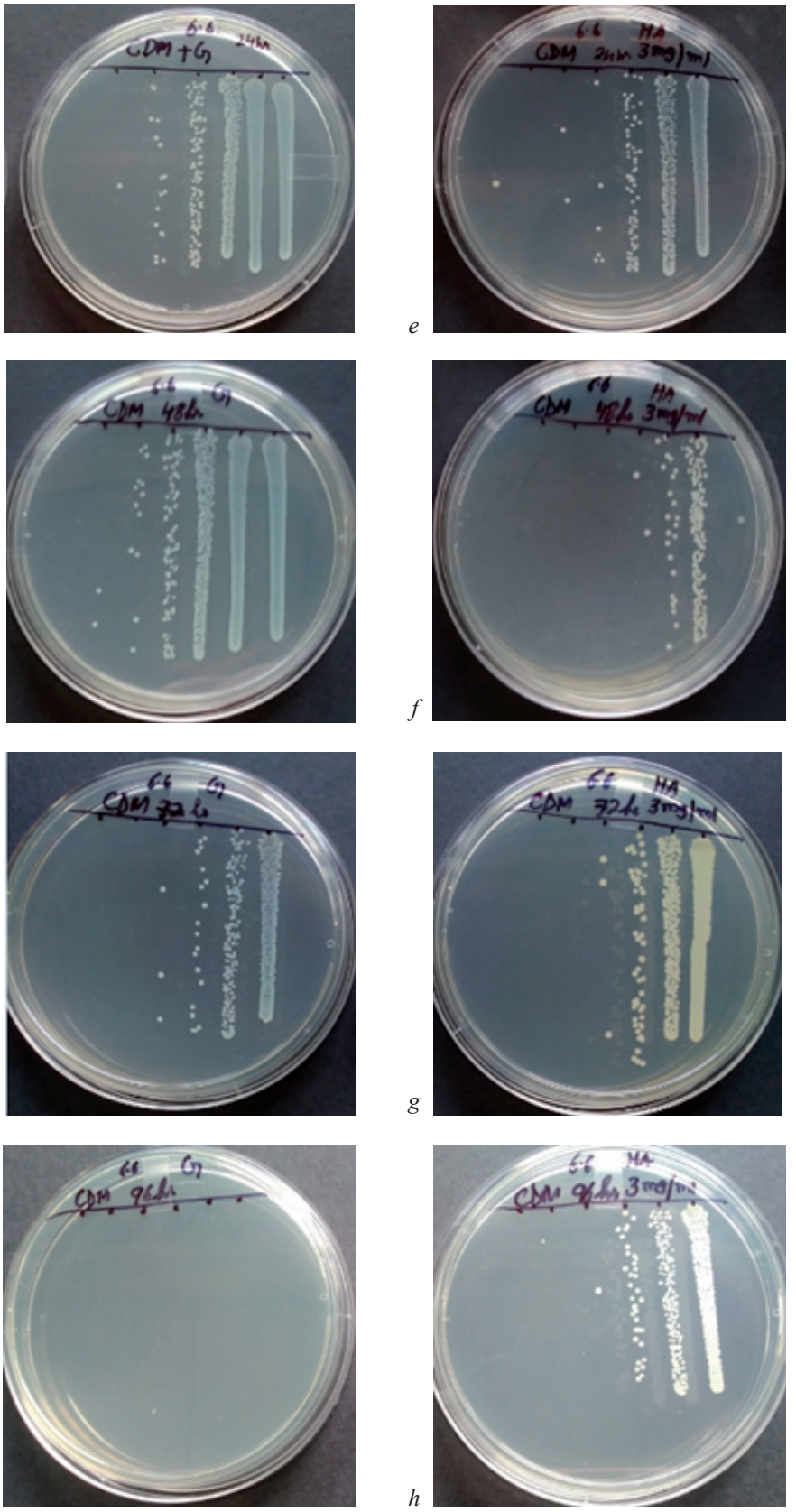


Fig. 2. Comparison of the growth of *P. multocida* in CDM containing glucose with CDM without glucose but hyaluronic as the sole carbon source

Table 1. Mean ± SE of CFU (millions/ml) $\log_{10}$ of *P. multoicda* growth at different time points

Incubation (h)	CDM + Glucose (CFU (millions/ml) $\log_{10}$)	CDM + Hyaluronic acid (CFU (millions/ml) $\log_{10}$)
24	2.82 ± 0.02	1.29 ± 0.01
48	2.90 ± 0.04 **	0.17 ± 0.03**
72	0.77 ± 0.02**	1.23 ± 0.02
96	0.00 ± 0**	1.12 ± 0.02**

Values are presented as mean ± SE. **Values in the row differ significantly at P<0.001

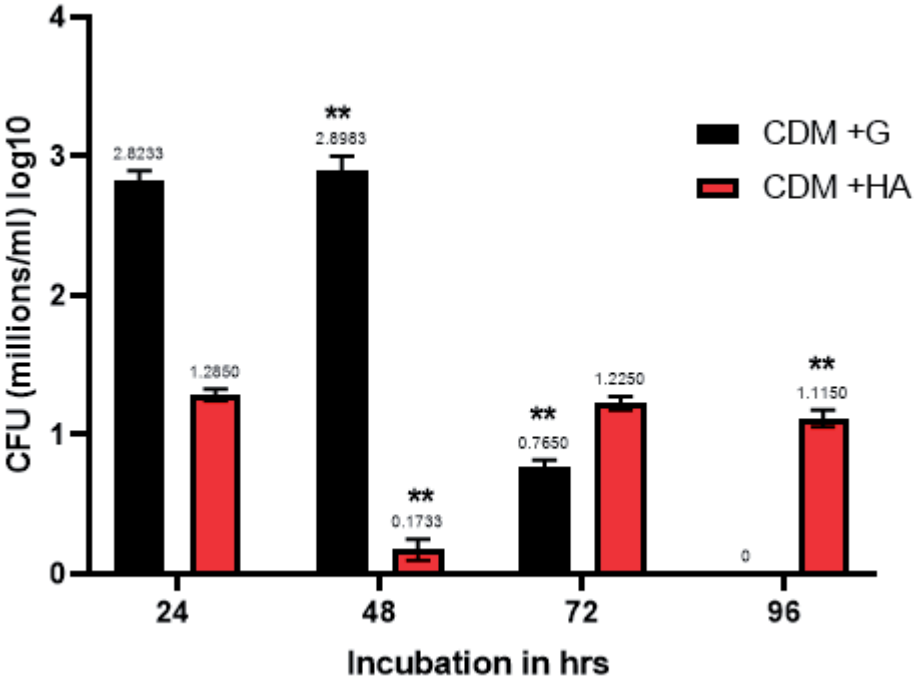


Fig. 3. Graphical representation of the utilisation of hyaluronic acid by *P. multocida* for sustained growth

Each bar represents the mean ± SDM., **P<0.05 (statistically significant differences between groups by two-way ANOVA with multiple comparison test). Growth assay of isolates presented as CFU (millions/ml) log10) at different incubation periods.

Discussion

Despite the fact that hyaluronic acid is essential for both structural and physiological features in alveolar gaps, its role in Pasteurella infection is unknown. In the present study the *P. multocida* P52 strain and 6 isolates were found to thrive on

a medium with hyaluronic acid as the sole carbon source. This is the first ever report of *P. multocida* B:2 using hyaluronic acid for growth.

In 1980, it was discovered that *P. multocida* B:2 produces hyaluronidase, and it was proposed as a virulence factor (CARTER and CHENGAPPA, 1980). Previous research has suggested that pathogenic bacteria produce hyaluronidase, which degrades hyaluronic acid and may aid in bacterial growth. Exogenous supplementation of hyaluronic acid, the substrate of hyaluronidase, was therefore tested to see if it could support *P. multocida* proliferation.

As hyaluronic acid is a long carbon chain, we assumed that cleavage would be required before it could be used as a carbon source and, as expected, hyaluronidase activity was detected in the *P. multocida* P52 strain and 6 other field isolates and *Staphylococcus aureus* (positive control), but not in E. coli (negative control). These findings are in agreement with CARTER and CHENGAPPA (1980) who examined four capsular serotypes, viz. A, B, D and E, of *P. multocida* and discovered that hyaluronidase production was restricted to *P. multocida* B:2. Other harmful gram positive and gram-negative bacteria, such as *Streptococcus, Staphylococcus*, and *Streptomyces,* have been demonstrated to produce hyaluronidases (HYNES and WALTON, 2000).

For the study of hyaluronic acid as a source of nutrients and for growth, we first evaluated the growth of *P. multocida* in chemically defined media that did not contain any carbohydrate but hyaluronic acid at different concentrations, viz .0. 5, 1, 3 and 5 mg/ml. Our findings demonstrated that hyaluronic acid at low concentrations (0.5mg/ml and 1mg/ml) did not supported the growth of *P. multocida* after 48 hours of incubation. It was also observed that a high concentration of hyaluronic acid (5 mg/ml) inhibited the growth of *P. multocida*, most likely due to the strong osmotic pressure created. However, continued growth of P. *multocida* was observed in the medium containing HA at a concentration of 3mg/ml even after 96 h of incubation. Depending on the bacteria, the concentration of HA can be inhibitory or supportive. In one study, for example, HA at 0.125-0.5 mg/ml promoted the growth of lactic acid bacteria, whereas higher concentrations (1 and 2 mg/ml) were inhibitory (DI CERBO et al., 2013). Another study found that HA at a concentration of 5 mg/ml aided the growth of *Staphylococcus aureus* (IBBERSON, 2015). In our study, lower concentrations of HA (0.5-1mg/ml) were found to be unsupportive for bacterial growth, whereas higher concentrations (5 mg/ml) were found to be inhibitory. Only 3mg/ml of HA was found to be adequate for bacterial growth.

In this study, *P. multocida* showed a decline in growth after 48 hours of incubation in the medium containing HA, indicating adaptation of *P. multocida* to hyaluronic acid. This also suggests that once adapted to hyaluronic acid growth, efficient digestion can occur, as evidenced by sustained growth. There was significant variation in the amount of growth of the six isolates, which could be attributed to variances in hyaluronidase production. After 48 hours of incubation, some isolates displayed changes in their colony's pigment, which could be due to hyaluronic acid adaption.

The experiment further suggests that *P. multocida* growth on hyaluronic acid is not as efficient as on simple carbohydrates. A similar finding has been reported by MARION et al. (2012), who observed reduced growth of *Streptococcus pneumoniae* when grown solely on hyaluronic acid as a carbon source. This thus implies that the kind and concentration of carbon sources has a substantial impact on cell growth. In agreement with this assertion, a high cell mass of *P. multocida* field isolates was reported when grown on sucrose, glucose and fructose (HUSSAIN et al., 2012).

Standard plate count and turbidity are the two commonly employed methods for measurement of bacterial growth. We preferred the former method to the latter, as turbidity measurements are subject to error when cultures contain fewer than 1 million cells per millilitre. Besides, turbidity in the bacterial culture could also be produced by the dead cells. In the standard plate count method, only the viable cells can grow and form colonies (BLACK and BLACK, 2014).

The respiratory airways are generally low in free sugar concentration (PHILIPS et al., 2003)

but fairly moderate in HA concentration (FRASER et al., 1997). It is thus possible that *P. multocida* may use hyaluronic acid for growth. The results of our experiment show that *P. multocida* could use hyaluronic acid as a carbon source. In nutritionally deprived conditions, this strategy could be used to obtain nutrients. The resultd suggest the role of hyaluronic acid in *P. multocida* infection. It is anticipated that the hyaluronidase enzyme, produced specifically by *P. multocida* serotype B:2, could facilitate its invasion (JEDRZEJAS, 2001). The observation that *P. multocida* can grow solely on hyaluronic acid shows that this enzyme could play a role in HS disease pathogenesis through several mechanisms, and that *P. multocida* may use hyaluronic acid as a carbon source during colonisation.

Conclusions

In conclusion, the study investigated the role of hyaluronic acid in *P. multocida* proliferation. The field isolates of *P. multocida* incubated on agarose plates with hyaluronic acid, produced hyaluronidase, which was demonstrated as a halo around the colonies owing to the degradation of hyaluronic acid. Further, the illustration of growth of *P. multocida* in the CDM containing hyaluronic acids as a sole carbon source is indicative of the utilisation of hyaluronic acid as a nutrient. It is suggested that sudden death in HS disease could be due to massive proliferation of *P. multocida* and the resultant release of endotoxin. In such circumstances, *P. multocida* may use hyaluronic acid as a nutrient in addition to glucose. Further study is needed to determine the role and significance of hyaluronidase in this pathogenesis using selective hyaluronidase inhibitors. Recently, L-Ascorbic Acid (an inhibitor of hyaluronidase) supplementation in mice infected with *P. multocida* has been demonstrated to lower bacterial burden and mortality. Whether L-Ascorbic Acid lessens the HS disease severity in bovine is yet to be ascertained.

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SAŽETAK

Pasteurella multocida B:2 (P. multocida) uzrokuje hemoragijsku septikemiju (HS), smrtonosnu bolest u goveda i bivola. Unatoč znatnoj proširenosti ovog patogena, vrlo se malo zna o čimbenicima koji pridonose njegovoj patogenosti. P. multocida proizvodi hijaluronidazu, enzim koji razgrađuje hijaluronsku kiselinu. Njegova uloga u patogenosti ove bakterije, međutim, nije poznata. Cilj je ovog istraživanja bio procijeniti potencijal bakterije P. multocida u iskorištavanju hijaluronske kiseline kao hranjive tvari in vitro. Istražen je rast šest izolata bakterije P. multocida izoliranih za vrijeme izbijanja HS-a u kemijski definiranom mediju (CDM) s glukozom i CDM-u bez glukoze s dodanom hijaluronskom kiselinom (HA). Bakterijski je rast procijenjen brojenjem kolonija 24, 48, 72 i 96 sati (h) nakon početka pokusa te je izražen u jedinicama CFU milijun/mL log10. P. multocida nastavila je rasti tijekom pokusa u kojemu je CDM-u dodan HA. U skupini u kojoj je CDM-u dodana glukoza rast je nastavljen 72 h od početka pokusa, nakon čega je uslijedio pad te nije bilo nikakva daljnjeg rasta. Ovo je prvo izvješće o bakteriji P. multocida koja se za svoj rast koristi hijaluronskom kiselinom, što bi se moglo iskoristiti za dobivanje hranjivih tvari za kolonizaciju i proliferaciju.

Ključne riječi: *P. multocida*; hijaluronidaza; hijaluronska kiselina; CDM