THE INVASIVENESS AND PERSISTENCE OF *Pasteurella multocida* AFTER INTRATRACHEAL INOCULATION INTO TURKEYS

Invasif dan Persisten Pasteurella multocida Setelah Diinokulasi Secara Intratracheal pada Kalkun

Mahdi Abrar dan Fakhrurrazi
Veterinary Faculty of Syiah Kuala University, Banda Aceh

ABSTRAK

The objective of this study was to evaluate the invasiveness and persistence of *Pasteurella multocida* after intratracheal inoculation on turkeys. Four strains of *Pasteurella multocida,* P-1059, T-325, CU, and M-9 were used in this study and intratracheally (IT) inoculated into turkeys. The number of viable bacteria in the trachea, lung, liver, spleen, and the blood were enumerated during periods of 6 and 9 hrs post inoculation (PI). The result showed that four strains of organism were present in the organ and the tissues observed. In the liver, spleen, and blood, the virulent P-1059 strain showed significant increases within 6 or 9 hrs PI. Strain CU showed significant increase only in the spleen. Two other strains did not show significant increase in any organs; strain M-9, on the other hand, showed significant decrease in the trachea. These results indicated that P-1059 and three other strains showed their ability to invade the tissues or organs when they are intratracheally inoculated.

ABSTRACT

Empat *Pasteurella multocida* strain*,* P-1059, T-325, CU, and M-9 telah dipelajari pada penelitian ini. Strain strain tersebut diinokulasikan secara intratrachea dan dihitung jumlah bakteri pada masing masing organ; trachea, hati, spleen, dan darah setelah 6 dan 9 jam inokulasi. Hasil penelitian ini menunjukkan bahwa keempat strain tersebut ditemui penyebarannya pada organ dan jaringan yang diamati. Pada hati, spleen, dan darah strain virulen P-1059 memperlihatkan peningkatan jumlah yang sangat tajam dalam waktu 6 dan 9 jam setelah inokulasi. Strain CU peningkatan jumlah hanya terlihat pada spleen sedangkan dua strain lainnya T-325 dan M-9 tidak menunjukkan peningkatan akan tetapi menunjukkan penurunan yang sangat tajam pada trachea. Penelitian ini mengindikasikan bahwa P-1059 dan tiga strain lainnya memiliki kemampuan menginvasi jaringan ketika diinokulasi secara intratrachea.

INTRODUCTION

Pasteurella multocida is a highly invasive bacterium which causes fowl cholera in turkeys, chicken, and other avian species. Death often results from septicemia, but in some bird, chronic disseminated pasteurellosis can occur (Rhoades and Rimler, 1997; Christensen et al., 1998; Glisson et al., 2003).

There are conflicting reports on whether or not *P.multocida* caused persistent bacteremia and whether the bacterial replication occurs intracellular or extracellular (Tsuji and Matsumoto, 1999). The site of bacterial replication and the associated tissue lesions have not been adequately described. The site of bacterial replication and the associated tissue lesions have not been adequately described. Muhairwa et al., 2000; Zhang et al., 2005; Snipes et al., 1990; Christiansen et al., 1992 reported that when *P. multocida* strain X-73 inoculated via the nasal cleft, the lung of infected birds revealed moderate to general infiltration of interstitial tissue with heterophiles and intravascular of this cell.

Pathogenesis of fowl cholera is poorly understood. The invasion of the organism occurs primarily through the upper respiratory tract mucosa (Rhoades and Rimler, 1999) or through the lower respiratory tract (Chung et al., 2001). The organism in the blood is rapidly cleared and localized in the liver and spleen. The virulent organism rapidly multiplies in these organs and is abruptly released again into the blood shortly before death of the host (Tsuji and Matsumoto, 1999; Townsend et al., 2001). Some attention has been focused on the initial adhesion and invasion of the organism. Boyce et al. (2006) demonstrated that when a low number of a virulent strain was endotracheally

the blood and spleen as early as 6 hrs after inoculation. Intraairsac inoculation of CU vaccine strain resulted in detection of the organism in the blood at 3 hrs.

The present study was designed to investigate the invasion and persisting ability of four strains of *P. multocida,* in various tissues after their intratracheal inoculation. The four strains were an encapsulated P-1059, non-encapsulated T-325 strain derived from P-1059, and two vaccine strains, CU and M-9.

MATERIALS AND METHODS

Experimental Turkeys

Medium white turkeys were maintained as a closed flock. Progenies of the flock were maintained in a brooder unit up to seven weeks of age, and moved to a concrete animal isolation unit with wood shavings on the floor.

Bacteria

Pasteurella. multocida strain P-1059 was originally obtained from Dr. K. R. Rhoad, National Animal Desease Center. Strain T-325 was a spontaneous mutant lacking capsule derived from P-1059 strain. Strain CU was obtained from Kee Vet Laboratory, Alabama. Strain M-9 was obtained from Dr. M. Jensen, Briham University, Utah. All strains were propagated on dextrose starch agar (DSA), harvested in brain heart infusion broth (BHI) and stored at -70 C.

Inoculation

Pasteurella multocida strains were recovered from frozen cultures to DSA and incubated at 41 C for 5 hrs (P-1059), or at 35 C for 8 hrs (M-9). Confluent growth was harvested with 5 ml of BHI broth/plate and enumerated for viable counts by plating out serial dilutions on DSA. The culture harvest of each strain diluted 1:10 in BHI was used as inoculums.

Intratracheal Inoculation

The inoculums were transferred into a 5 ml syringe and polyethylene tubing (1.19 mm in inner diameter) in 5.1 cm length was attached on 18 ga. needle. The tubing was carefully inserted through the laryngeal cleft, and 2.5 cm below the cleft, the inoculums in 1 ml was delivered slowly drop wise. After inoculation, the head was held with the mouth open for 1 minute to prevent immediate expulsion of

Sampling Procedures

The turkeys were killed by electrical shock. A sterile cotton swab was inserted through the palatine cleft and streaked onto Mac Conkey agar. The shin of the neck was pulled back and the trachea was exposed and a ring of 1 cm width was cut for tracheal sample. For the rest of isolation, blood vessels were clamped off using hemostats, and the heart, liver, and digestive tract were lifted out. A portion of liver and spleen were removed. The right and the left lungs were removed. The entire tissue samples were placed in sterile plastic bags and immediately place on ice.

Sample Processing

Blood and tissue samples were immediately processed as follows; the blood was transferred to a sterile tube and mixed well. Serial 1:10 dilutions were made in 0.05 M phosphate buffer saline (PBS) by transferring 0.2 ml of blood to 1.8 ml of sterile PBS. The appropriate dilution in 0.1 was spread out onto DSA plate and incubated. Liver, spleen, and the right/left lungs were weight. Sterile PBS in 9 ml was added for every 1 gr of tissue to make the original 1:10 dilution. Serial 1:10 dilutions were made and samples plated out by the same method as for blood. Colony counts were made on all plate on the following day.

Experiment 1: Ten 8-week-old turkeys were inoculated intratracheally (IT) with 1,2 $x10^9$ CFU of P-1059. Five of them were terminated at 6 or 9 hrs post inoculation, and their tissues were examined for bacterial counts. Two uninoculated control birds were also processed. Experiment 2: Ten 8-week-old turkeys were inoculated intratracheally (IT) with 1,2 $x10^9$ CFU of *P. multocida* strain T-325 per bird. Other procedures were done in a same manner as in Experiment 1. Experiment 3: Ten 8-week-old turkeys were inoculated intratracheally (IT) with 1,2x 10^9 CFU of *P. multocida* strain CU per bird. Other procedures were done in a same manner as in Experiment 1. Experiment 4: Ten 8-week-old turkeys were inoculated intratracheally (IT) with 1,2$x10^9$ CFU of *P. multocida* strain M-9 per bird. Other procedures were done in a same manner as in Experiment 1.

Statistical Analysis

The colony counts were transformed into $\log_{10}$ values, and the mean and standard deviation were calculated in each group. Bacterial concentration for the two interval times (6 and 9

RESULTS AND DISCUSSION

Experiment 1. Turkeys were IT inoculated with $1.2x10^9$ CFU of P-1059 and tissues were examined for bacterial counts at 6 hrs and 9 hrs post inoculation (Figure I). No *pasteurella* was isolated from any tissue of the two uninoculated control turkeys. All the birds showed evidence of *Pasteurella* infection at 6 hrs PI; the organism was isolated in high numbers in respiratory tissues and in moderate numbers in the liver, spleen, and blood. At 9 hrs PI all birds showed high numbers of the organism in all tissue samples. Between 6 and 9 hrs PI, there was a highly significant ($P<0.01$) increase in the number of the organisms isolated from the liver, spleen, and blood (Table 1). The data indicate that P-1059 strain invaded systemically before 6 hrs PI followed by the rapid multiplication in the liver and spleen.

Experiment 2. Turkeys were IT inoculated with non-encapsulated T-325 strain and examined at 6 and 9 hrs PI (Fig II). The two uninoculated controls birds showed negative isolation results with any tissues. At 6 hrs PI, the bacteria were detected in high numbers in the respiratory tissues and also in the systemic organs. However, the number of organisms detected in the liver, spleen, and blood were smaller than those observed with P-1059 strain. Three out of the five birds showed negative results with blood samples. At 9 hour PI, T-325 strain did not show the vigorous multiplication in the systemic organs as seen with P-1059 strain. In fact, the number of organism in blood and liver, did not showed significant difference ($P>0.05$) between 6 and 9 hrs sampling time (Table I). One turkey showed less than 10 CFU/g in the blood at 9 hrs PI.

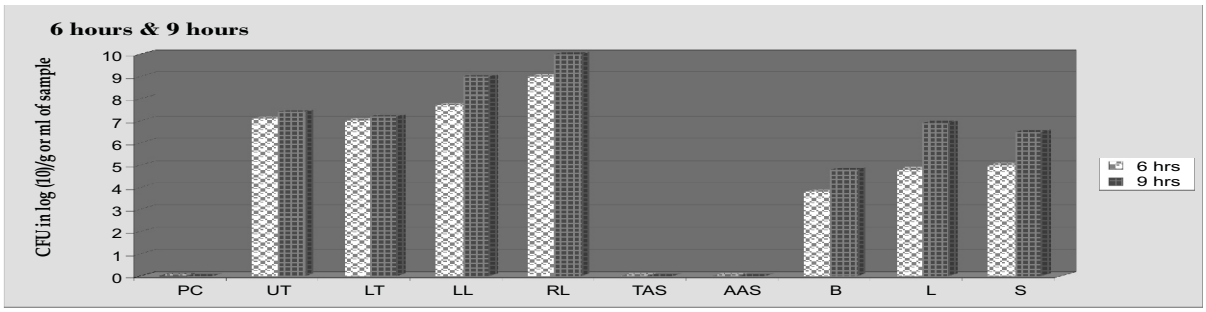

Figure 1. Bacterial recovery from the palatine cleft (PC), upper trachea (UP), left lung (LL), right lung (RL), thoracis airsac (TAS), abdominal airsac (AAS), liver (L), and spleen (S) of inoculation turkeys at 6 or 9 hrs after intratracheal (IT) inoculation with encapsulated P-1059 strain

Table 1. Summary of the statistical analysis using two sample comparison test based on $H_0: \mu_{6\,hrs} = \mu_{9\,hrs}$ for each strain.

	P. multocida strain			
Tissue	P-1059	CU	T-325	M-9
Upper Trachea	-	-	-	**
Lower Trachea	-	-	-	**
Left Lung	-	-	-	-
Right Lung	-	-	-	*
Blood	**	-	-	**
Liver	**	-	-	-
Spleen	**	*	*	-

**) significant *different* at 1% level, *) Significant *different* at 5% level, -) no significant *different* at 5% level

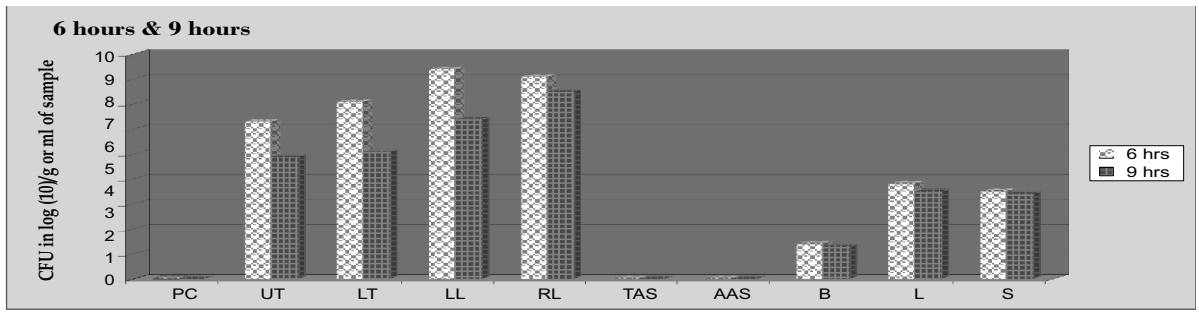

Figure 2. Bacterial recovery from the palatine cleft (PC), upper trachea (UP), left lung (LL), right lung (RL), thoracis airsac (TAS), abdominal airsac (AAS), liver (L), and spleen (S) of inoculation turkeys at 6 or 9 hrs after intratracheal (IT) inoculation with non encapsulated T-325 strain

Experiment 3. Strain CU was IT inoculated and tissues were examined at 6 and 9 hrs PI (Figure III). No *pasteurella* was isolated from any tissues of the two control turkeys. At 6 hrs PI the organism was abundant in respiratory tissues similar to the observation with P-1059 or T-325 strain. As with the case of T-325, no significant ($P>0.05$) increase of the organism was seen in the blood or liver. In the spleen however, a significant ($P<0.05$) increase in the bacterial number was observed.

Experiment 4. Turkeys were IT inoculated with M-9 strain and tissues were examined at 6 and 9 hrs PI (Figure 4). No organism was isolated from any tissue of the two uninoculated control birds. The organism was isolated in moderate numbers in the respiratory tissues at 6 hrs PI. Unlike the three other strains, significantly fewer ($P<0.05$) organism were detected at 9 hrs than 6 hrs PI in the upper and lower trachea, the right lung, and blood. At 6 hrs PI, 4 turkeys showed negative isolation with the blood, 1 with liver, and 1 with spleen. At 9 hrs PI, 3 out 5 turkeys showed negative result with blood, and 1 out 5 with spleen.

Fowl cholera is causes by strains of *Pasteurella multocida* that belong to capsular type A and various somatic types. The organisms isolated from turkeys predominantly belong to type 3 or type 3, 4 (Harper et al., 2007). However, it is generally accepted that serotype specificity has no correlation with virulence of organism. Thus, among strains belonging to somatic type of 3, 4, there are very virulent strains and those of low virulence; the latter include M-9 vaccine strain (Huber et al., 2002; Hunt et al., 2000). In this study, we examined the invasiveness and persistence of virulent or vaccine strain *P. multocida* after their intratracheal inoculation into turkeys and found that significant difference among strains was detected in their persistence in various tissues but not their invasiveness. In the present investigation, we compared two strains, a virulent P-1059 and M-9 vaccine strain, in their pathological responses after their IT inoculation into turkeys.

In the present study, high numbers (10^9 CFU) of viable *P. multocida* were deposited on the surface of tracheal mucosa. Our preliminary study indicated that even a virulent P-1059

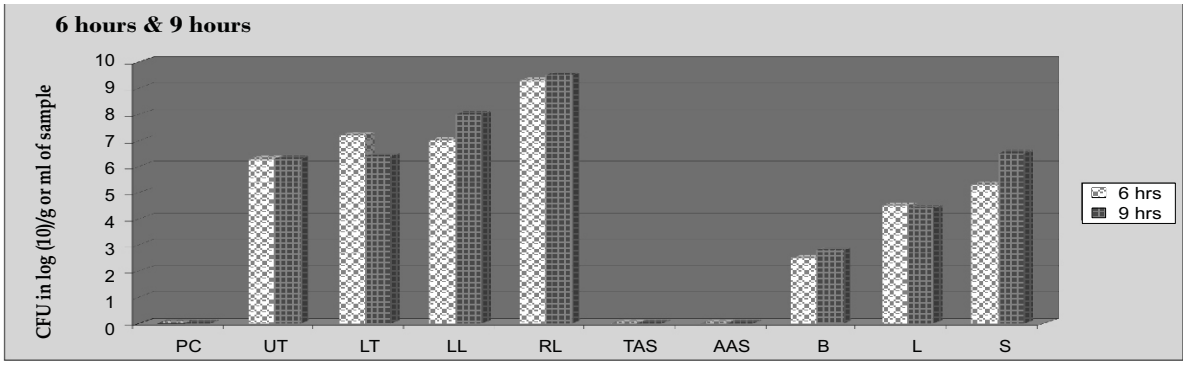

Figure 3. Bacterial recovery from the palatine cleft (PC), upper trachea (UP), left lung (LL), right lung (RL), thoracis airsac (TAS), abdominal airsac (AAS), liver (L), and spleen (S) of inoculation turkeys at 6 or 9 hrs after intratracheal (IT) inoculation with CU strain

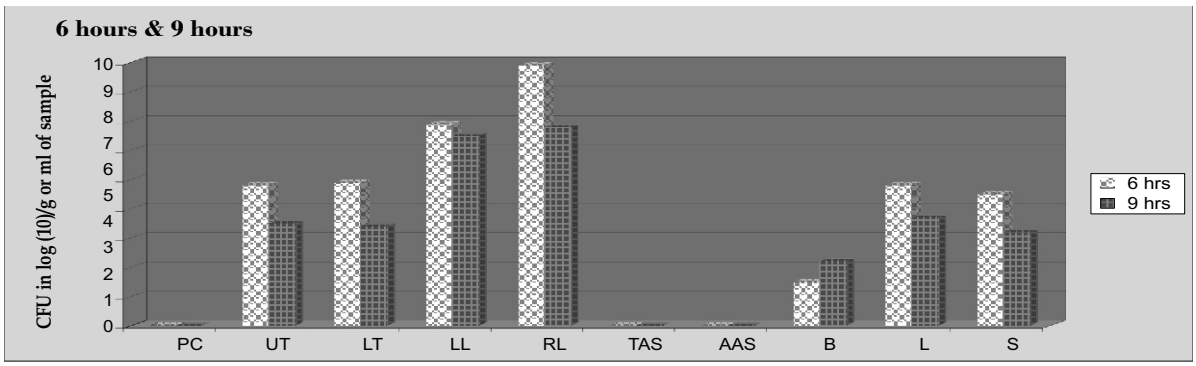

Figure 4. Bacterial recovery from the palatine cleft (PC), upper trachea (UP), left lung (LL), right lung (RL), thoracis airsac (TAS), abdominal airsac (AAS), liver (L), and spleen (S) of inoculation turkeys at 6 or 9 hrs after intratracheal (IT) inoculation with M-9 strain

strain caused systemic infection at a low rate when it was IT inoculated in lower than 10^8 CFU. Processing each tissue for accurate CFU counts technically limited us to examine 12 birds in each experiment. The combination of these two factors was the reason for the use of high inoculums amount to pursue the main objective; evaluating invasiveness of the four strains after IT inoculation.

Pasteurella multocida is suggested to invade the upper respiratory mucosa to reach the blood stream (Rhoades and Rimler, 1991). When virulent P-1059 strain was swabbed at the palatine cleft, however, no systemic infection was detected at 6 hrs postinoculation (Rhoades and Rimler, 1997; Wilkie et al., 2000). Intratracheal inoculation of P-1059 strain resulted in the detection in the liver as early as 3 hrs post inoculation. A live vaccine strain, CU, was detected in the blood at 3 hrs after the inoculation of the organism into the air sac (Ficken and Barnes, 1989).

When P-1059 strain was IT inoculated into turkeys, it multiplied *in situ,* spreading gradually downwards along the airway in a majority of the animals, while in some animals, the organism invaded the blood and systemic organs in less than one hour (Tsuji and Matsumoto, 1999). In the present study, P-1059 and three other rains showed their high rate of invasiveness when they were IT inoculated in high numbers, suggesting that there is not a significant difference among the four strains in their capacity to cause systemic invasion from the respiratory tract in the turkeys. The four strains, however, showed differences in their capacity to multiply in various organs.

The statistical analysis shown in Table 1 indicates there are differences among strains in their capacity to multiply in various organs. Between 6 and 9 hrs of isolation, P-1059 strain showed significant increase in bacterial numbers in the blood, livers, and spleen. Strain CU showed a significant increase only in the spleen. The other two strains did not show a significant increase in any organs. Strain M-9, on the other hand, showed significant decrease between the two isolation attempts in the upper and lower trachea, right lung and the blood. Two factors should be considered for the cause of these differences. Strain P-1059 and CU are encapsulated, while T-315 and M-9 are not or poorly encapsulated; P-1059 or T-325 grows optimally at 41 C, while the two vaccine strains are temperature-sensitive mutants, growing

Pathogenesis and immunity on fowl cholera are poorly understood. The organism invades from the respiratory tract, enters the bloodstream, and localizes in the liver and spleen. Virulent organisms multiply rapidly in these organs, while those belonging to low virulent strains are killed at various rates (Boyce et al., 2009). In immune turkeys, a virulent strain is killed in the liver (Tsuji and Matsumoto, 1990). The mechanism by which the organism is killed in the liver is not known. The results of the present study generally support these pathogenic processes. In addition, the results indicate that both virulent and current vaccine strains all possess high capacity to invade from the respiratory tract into the bloodstream.

CONCLUSIONS

The fate of four strains of *Pasteurella multocida* was studied after their intratracheal inoculation in young adult turkeys. Viable bacterial counts were made in respiratory tissues as well as in the liver, spleen, and blood at 6 and 9 hrs after the inoculation of approximately 10^9 viable organisms of each strain. A virulent, encapsulated strain, P-1059, invaded systemically by 6 hrs post inoculation (PI) and multiplied vigorously in all tissues and organs examined. A blue colony mutant of P-1059, T-325, which does not possess a thick layer of capsule, as well as CU vaccine strain, invaded the parenchymal organs, but did not show significant increase in viable counts at 9 hrs PI compared with at 6 hrs PI. The results indicate that all the four strains possess high capacity to invade respiratory tissues with varying capacity to persist in host tissues.

REFERENCES

Boyce, J. D., P.A. Cullen, V. Nguyen, I. Wilkie, and B. Adler. 2006. Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the in vivo environment of the natural host. **Proteomics**. *6* (3): 870-880.

Boyce, J.D., D. John, W. Wilkie, W. Ian, F. Mark, J. Cox, and D. Andrew. 2009**.** Identification of novel glycosyltransferases required for assembly of the *Pasteurella multocida* A:1 Lipopolysaccharide and their involvement in virulence. **Infection and**

Chung, J.Y., I. Wilkie, J.D. Boyce, K.M. Townsend, A.J. Frost, and M. Ghoddusi. 2001. Citation Role of capsule in the pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A.: Infect-immun. Washington, D.C., **American Society for Microbiology**. 69 (4):2487-2492.

Christensen, J.P., H.H. Dietz, and M. Bisgaard. 1998. Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from back-yard poultry and from two outbreaks of avian cholera in avifauna in Denmark. **Avian Pathol.** 27:373-381.

Christiansen, K.H., T.E. Carpenter, K.P. Snipes, and D.W. Hird. 1992. Transmission of *Pasteurella multocida* on Californian turkey premises in 1988–89. **Avian Dis.** 36:262–271.

Glisson, J.R., C.L. Hofacre, and J.P. Christensen. 2003. Fowl cholera. *In* **Diseases of Poultry**. Saif, Y.M., H.J. Barnes, and J.R. Glisson (eds). 11th ed. Iowa State University Press, Ames.

Ficken, M.D. and H.J. Barnes. 1989. Airsaculitis in turkeys inoculated with *Pasteurella multocida*, **Vet. Pathol.** 26:231-237.

Harper, M, J. Boyce, D. John, N. Cox, D. Andrew; St. Michael, Frank, W. Wilkie, W. Ian, P.J. Blackall, and B. Adler. 2007. *Pasteurella multocida* expresses two lipopolysaccharide glycoforms simultaneously, but only a single form is required for virulence: Identification of two acceptor-specific heptosyl I transferases. **Infection and Immunity**, *75* 8: 3885-3893.

Huber, B.S., D.V. Allred, J.C. Carmen, D.D. Frame, D.G. Whiting, J.R. Cryan, T.R. Olson, P.J. Jackson, K. **Hill, M.T. Laker, and R.A. Robison.** 2002. Random amplified polymorphic DNA and amplified fragment length polymorphism analyses of *Pasteurella multocida* isolates from fatal fowl cholera infections. **J. Clin. Microbiol**. 40:2163-2168.

Hunt, M.L., B. Adler, and K.M. Townsend. 2000. The molecular biology of *Pasteurella multocida*. **Vet. Microbiol**. 72:3-25.

Muhairwa, A.P., J.P. Christensen, and M. Bisgaard. 2000. Investigations on the carrier rate of *P. multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera. **Avian Pathol**. 29:133-142.

Rhoades, K.R. and R.B. Rimler. 1997. *Pasteurella multocida* colonization and invasion in experimentally exposed turkey. **Avian Dis.** 34:381-383.

Snipes, K.P., D.C. Hirsh, R.W. Kasten, T.E. Carpenter, D.W. Hird, and R.H. McCapes. 1990. Homogeneity of characteristics of *Pasteurella multocida* isolated from turkeys and wildlife in California. **Avian Dis**. 34:315-320.

Townsend, K.M., J.D. Boyce, J.Y. Chung, A.J. Frost, and B. Adler. 2001. Genetic organization of *Pasteurella multocida cap* loci and development of a multiplex capsular PCR typing system. **J. Clin. Microbiol**. **39:**924-929.

Townsend, K.M., J.D. Boyce, J.Y. Chung, A.J. Frost, and B. Adler. 2001. Genetic organization of *Pasteurella multocida cap* loci and development of a multiplex capsular PCR typing system. **J. Clin. Microbiol**. 39:924-929.

Tsuji, M. and M. Matsumoto. 1999. Pathogenesis of fowl cholera: Influence of encapsulation on the fate of *Pasteurella multocida* after intravenous inoculation into turkeys. **Avian Dis**. 32:9-15.

Wilkie, W., O. Boyle, and A. Frost. 2000. The virulence and protective efficacy for chickens of *Pasteurella multocida* administered by different routes. **Veterinary Microbiology**. *72*:57-68.

Zhang, N., Fegan, I. Fraser, P. Duffy, R.E. Bowles, A. Gordon, P.J. Ketterer, W. Shinwari, and P.J Blackall. 2005. Molecular epidemiology of two fowl cholera outbreaks on a free-range chicken layer farm. **Journal of Clinical Microbiology**. 43(6):2959-2961.

UCAPAN TERIMA KASIH

Ucapan terima kasih dan penghargaan yang tinggi diberikan kepada Mitra Bestari yang telah terlibat dalam menelaah artikel pada Jurnal Kedokteran Hewan Volume 4 No. 2 Tahun 2010. Berikut ini adalah nama-nama Mitra Bestari yang berpartisipasi:

Prof. Dr. drh. Aulanni'am, DES
Fakultas MIPA Universitas Brawijaya

Prof. (emeritus) Dr. Slamet Subagyo
Fakultas Kedokteran Hewan Universitas Gadjah Mada

Prof. Dr. drh.Pratiwi Trisunawati, MS
Program Kedokteran Hewan Universitas Brawijaya

Dr. drh. I Wayan Batan, MS
Fakultas Kedokteran Hewan Universitas Udayana

Prof. Dr. drh. Aulanni'am, DES
Fakultas Kedokteran Hewan Institut Pertanian Bogor

Prof. Dr. Dr. Sumarno, Sp.MK
Fakultas Kedokteran Universitas Brawijaya

Ir. Diah T. Widayati, MP., Ph.D
Fakultas Peternakan Universitas Gadjah Mada

Dr. drh. Pantja Madyawati, M.Si
Fakultas Kedokteran Hewan Universitas Airlangga

drh. Lilik Kusindarta, MP., Ph.D
Fakultas Kedokteran Hewan Universitas Gadjah Mada

Dr. drh. Widjiati, M.Si
Fakultas Kedokteran Hewan Universitas Airlangga

Dr. drh. Bayyinatul Muchtaromah, MS
Fakultas MIPA Univeritas Islam Negeri Malang

Dr. drh. Sri Murwani, MP
Fakultas Kedokteran Universitas Brawijaya

INDEKS PENULIS

Author Index

INDEKS JUDUL

JURNAL KEDOKTERAN HEWAN
Volume 4 (No. 1-2), Tahun 2010

Edisi Maret 2010

Edisi September 2010

ISSN : 1978-225X

JURNAL KEDOKTERAN HEWAN

Terbit setiap Maret dan September

DAFTAR ISI

JKH	Vol. 4	No. 2	Hal 49-98	Banda Aceh, September 2010	ISSN: 1978-225X

Jl. Tgk. Hasan Krueng Kalee No. 4 Kampus FKH Unsyiah
Darussalam Banda Aceh, Aceh
Telp./Fax. 0651-7551536
E-mail : jurnal_khusk@yahoo.com

ISSN 1978-225X
9 771978 225061