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Adherence of *Gallibacterium anatis* to Inert Surfaces

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Abstract: The genus Gallibacterium includes bacteria of avian origin isolated from a variety of birds. It has been described as an indigenous bacterium to the upper respiratory and lower genital tracts of healthy chickens but it has been also reported associated to different pathological conditions. Microbial infections are initiated by colonization of tissues by a specific mechanism of adherence to host cells. In this research, we evaluated the ability of the *G. anatis* strains F149^T, 12158/5 Salp and the *G. genomospecies* 1 strain CCM 5974 to adhere to inert surfaces as a possible mechanism promoting biofilm formation. All three strains formed robust biofilms on polystyrene and glass. Adherence was prevented by treating bacterial cells with trypsin, suggesting the participation of proteins in this process. Scanning electron microscopy of *G. anatis* adherence to glass surface was observed within the 1st 3 h of exposure. Extracellular material, micro-vesicles, filamentous structures and cords were observed as a part of biofilm matrix. The adhesive capacity observed could be an important ability for colonization of tissue surfaces and for allowing Gallibacterium to persist inside its host.

Key words: *G. anatis*, adhesion, biofilm, bacterial cells, mechanism, surface

INTRODUCTION

The genus Gallibacterium, recently incorporated within the family Pasteurellaceae includes bacteria of avian origin once known as *Pasteurella haemolytica*, *Actinobacillus salpingitidis* and *Pasteurella anatis* (Christensen *et al*., 2003). Within the *Gallibacterium anatis* species, two biovars haemolytica and anatis are currently recognized. *Gallibacterium* sp. has been isolated from a variety of birds including chickens, turkeys and ducks among others.

Gallibacterium anatis is considered to be a part of the normal bacterial flora of the upper respiratory tract and the lower genital tract but has also been reported associated to pathological conditions such as salpingitis, peritonitis, septicemia, pericarditis, hepatitis, enteritis and respiratory tract lesions (Addo and Mohan, 1985; Bisgaard, 1977; Mushin *et al*., 1980; Neubauer *et al*., 2009; Shaw *et al*., 1990). As a potential pathogen, Gallibacterium must have different virulence factors enabling colonization, invasion and means avoiding the host immune response. Until now, only secretion of IgG degrading proteases (Garcia-Gomez *et al*., 2005), the capability of agglutinating red blood cells (Zepeda *et al*., 2009) and production of a cytolytic RTX-toxin (Kristensen *et al*., 2010) have been described for this microorganism. Other possible virulence factors, including colonization factors, remain to be described.

It has been shown that the ability of bacteria to form adherent biofilms on inert surfaces is correlated with ability to cause infections *in vivo* (De Oliveira-Garcia *et al*., 2003; Fey and Olson, 2010; Rupp and Archer, 1992). A biofilm has been considered as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al*., 1999). However, until now only the capability of *G. anatis* to adhere to chicken tracheal or oviduct epithelial cells has been reported by

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Ramirez *et al.* (2007). To extend the insight into mechanisms *G. anatis* uses for colonization, we aimed at evaluating the capability of three different strains of *G. anatis* to adhere to inert surfaces (plastic or glass) as a possible mechanism promoting biofilm formation *in vivo*.

MATERIALS AND METHODS

Bacterial isolates and growth conditions: The Gallibacterium strains included in this research were the *G. anatis* biovar *anatis* (F 149), *G. anatis* biovar *haemolytica* (12158/5 Salp) and *G. genomospecies* 1 (CCM5974^T), respectively (Mraz *et al.*, 1976; Zepeda *et al.*, 2009). Strain F149^T was isolated from the intestinal tract of a healthy duck whereas 12158/5 Salp was isolated from a layer chicken with salpingitis and CCM5974^T was isolated from the liver of a layer chicken that died from septiceamia, respectively.

Media: Bacteria were cultivated on 5% sheep blood agar (BD Bioxon, Becton Dickinson, Cuautitlan Izcalli, Mexico, Mexico) at 37°C and incubated overnight in a incubator with CO_2 (Lab-line). Brain-heart infusion broth (BHI; BD Bioxon) was used for propagation of bacterial cultures.

Microtitre plate adherence assay: The three *G. anatis* strains were cultured overnight in BHI and centrifuged for 2 min at 12.000 g. Cell pellets were washed and resuspended in phosphate-buffered saline (PBS, pH 7.2) to obtain an Optical Density (OD) of 1 at 600 nm. In order to determine bacterial microtitre plate adherence, wells of sterile 96-well U-bottomed polystyrene microtitre plates (Sarstedt, Barcelona, Spain) were filled with 180 μL of BHI medium and inoculated with 20 μL of standardized *G. anatis* cell suspensions in triplicate.

Negative control wells containing only broth or PBS were included in each assay while *Staphylococcus epidermidis* ATCC 35984 was used as positive control. Plates were incubated under stationary conditions at 37°C for 24 h.

The liquid contents of each well were then aspirated and the wells were washed three times with 250 μL of sterile PBS. Finally, the adherent cells were fixed with 200 μL of methanol for 15 min. After air-drying, wells were stained with 150 μL of 2% Hucker's crystal violet for 5 min. Dye bound to adherent cells was re-solubilised with 150 μL of 33% (v/v) glacial acetic acid and the OD of the re-solubilised dye of each well was obtained at 630 nm using an automated microtitre plate reader (Multiscan FC; Thermo Labsystems, Helsinki, Finland).

Adherence quantifications were conducted in triplicate on three separate occasions and the results averaged (Fredheim *et al.*, 2009). In order to determine if some protein components participated in the adherence to the plastic surface, bacteria were incubated in the presence of 1 mg mL^{-1} trypsin. After incubation, plates were processed as above and re-solubilized crystal violet was quantified. To determine if the exuded material was of polysaccharide nature, bacterial films obtained over a cover glass were stained with alcian blue, a cationic dye that stains sulphated mucopolysaccharides or glycosaminoglycan anionic polysaccharides (Pompilio *et al.*, 2008).

Ultrastructural studies: An overnight culture of F149^T, 12158/5 or CCM5974 in BHI medium was used to inoculate glass coverslips inside 60×15 plastic plates (1% inoculum v/v). Plates were incubated at 37°C during 3, 6, 12, 24 or 48 h. Afterwards, bacteria on the glass coverslips were fixed with 2.5% glutaraldehyde during 1 h, washed 3 times with 25 mM Tris-HCl, pH 8.0; post-fixed with 1% osmium tetroxide during 1 h, washed 3 times and dehydrated in an ascending ethanol series (50, 60, 70, 80, 90 and 100%) before critical-point drying. Specimens were examined with a JEOL JSM-6510LV Scanning Electron Microscopy (SEM) at 25 kV.

RESULTS AND DISCUSSION

Bacterial adherence to mucosal surfaces is the 1st step in the infectious process. Thus, successful adherence could promote colonization of different tissues inside a host. One way of promoting persistent infections is via biofilm formation which often seems to be involved in chronic infectious diseases (Vu *et al.*, 2009). *Gallibacterium anatis* appear in particular to be associated to infections of the reproductive tract and the peritoneum in chickens which may result from an ascending infection from the cloaca where *G. anatis* is found very frequently in healthy birds (Bojesen *et al.*, 2003; Neubauer *et al.*, 2009).

To overcome the propulsive effect of peristalsis leading preventing *G. anatis* from colonizing the upper reproductive tract, different means of attachment are likely involved. Attachment to both inert and living surfaces is a common property exhibited by many bacteria and is considered a 1st step in biofilm formation (Webster *et al.*, 2006). An important virulence factor exhibited by several bacterial pathogens is adhesion to epithelial and inert surfaces (Costerton *et al.*, 1999). Although, *Gallibacterium anatis* adherence to epithelial cells was previously described by Ramirez *et al.* (2007), the capability of *Gallibacterium* to adhere to inert surfaces as an initial step in biofilm formation has not been

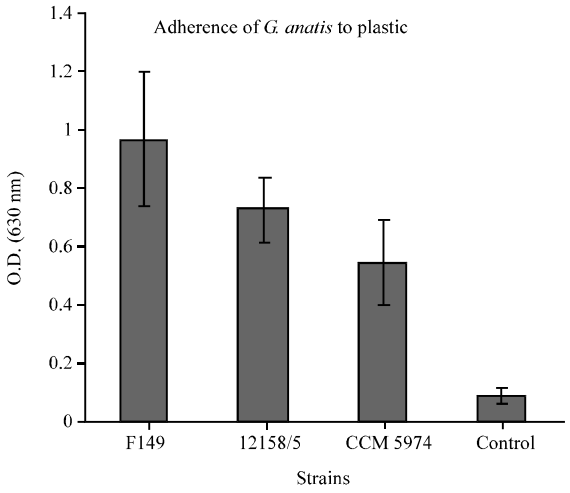

Fig. 1: Biofilm formation by *G. anatis*. Results are represented as mean OD_{630} value±SEM (Standard Error of the Mean) after crystal violet staining of biofilm in the plastic adherence assay. Values correspond to at least three different assays in triplicate

investigated and was therefore evaluated in this research. As can be seen in Fig. 1, the three strains formed robust biofilms ($F149^T$, 12158/5 Salp and CCM5974) with no statistical difference between the three. All three strains also formed biofilms on glass (Fig. 2). It has been shown that both, pathogenic and commensal, isolates of *Histophilus somni* are capable of forming biofilm although the pathogenic ones formed a more robust biofilm (Sandal *et al*., 2007). In contrast, it has been reported that generally, non-virulent serovars of *Haemophilus parasuis* showed a higher degree of *in vitro* biofilm formation than virulent serovars (Jin *et al*., 2006). As shown in Fig. 1, there was no correlation between the adherence capability to polystyrene of the *G. anatis* strains tested and their lesion-associated origin; since both commensal ($F149^T$) and lesion-associated (12158/5 Salp and CCM 5974) strains were able of adhering to plastic and glass surfaces at a similar level.

When the strains were incubated in the presence of trypsin attachment was not observed to plastic surface as OD_{630} values were similar to those obtained from negative controls (0.12) whereas the level of bacterial growth was

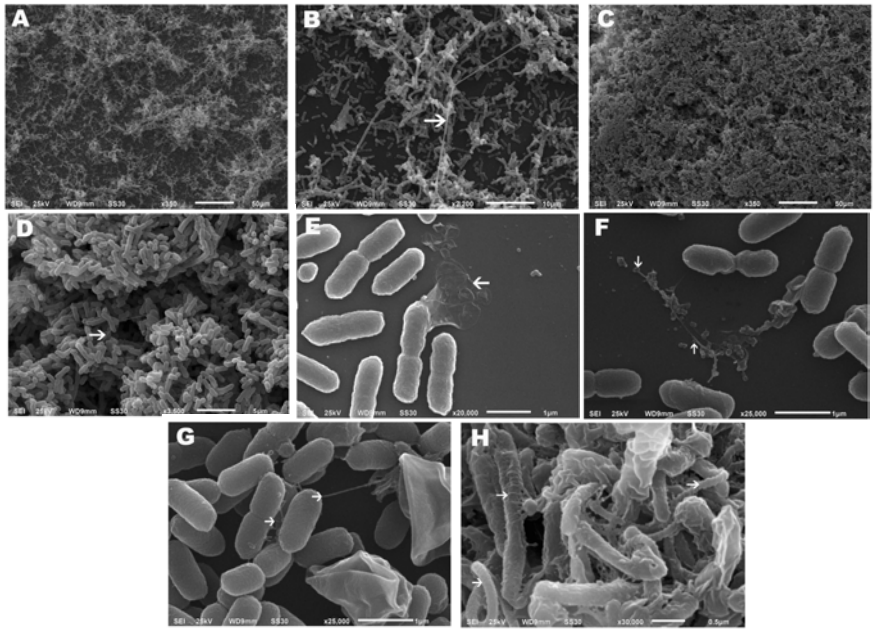

Fig. 2: Scanning electron microscopy images showing the adherence of *G. anatis* to glass and different components taking part in biofilm formation. *G. anatis* strains $F149^T$ (images A-D); CCM 5974 (E-G) and 12158/5 (H) were used for SEM observations. Images A and B correspond to samples of 12 h of incubation; C and D to 48 h; E and F to 3 h; G to 6 h and H to 48 h. *G. anatis* biofilm formation observed at low amplification (A-D). Extracellular products released into the medium by different bacteria (arrow, E). Extracellular products associated to microvesicles (arrows, F). Long (B, D and G) and short filamentous structures (G and H) joining close or separated bacteria (arrows) Filamentous cords are also observed as part of the biofilm complexity (H)

similar to that reached without trypsin, suggesting that proteins participate in the adhesion to a plastic surface. Biofilm formation is typically a multistep process that usually requires participation of structural appendages such as flagella, type IV pili and hemmaglutinins (De Oliveira-Garcia *et al*., 2003; O'toole and Kolter, 1998; Pompilio *et al*., 2008; Rupp and Archer, 1992), other bacterial proteins (Rohde *et al*., 2005; Webster *et al*., 2006), outer membrane vesicles (Yonezawa *et al*., 2009) and extracellular DNA (Fredheim *et al*., 2009).

Although, adhesion to ephitelial cells (Ramirez *et al*., 2007) and the capability to agglutinate red blood cells has been described (Zepeda *et al*., 2009) for Gallibacterium, no surface appendices such as flagella, fimbria or proteins, involved in adhesion have been described. However by transmission electron microscopy we have observed type IV pili-like or short fimbria on the surface of distinct *G. anatis* strains including F149^T (unpublished data). We speculate if these proteinaceous structures could be involved in *G. anatis* biofilm formation as has been described in other bacteria sharing this capability and because treatment with trypsin prevented adhesion without reducing growth.

It has been shown that proteins and extracellular DNA are more important than polysaccharide intercellular adhesion in *Staphylococcus haemolyticus* biofilm formation because biofilm treatment with proteinase K or DNAse produce a detachment of biofilms in 98 and 100%, respectively. On the contrary, treatment with NaIO4 that degrade β-1, 6-linked polysaccharides only produces a 38% detachment whereas, 90% *S. epidermidis* biofilm detachment was observed after treatment with NaIO4 (Fredheim *et al*., 2009). The biofilm forming ability of all three Gallibacterium strains was evaluated by SEM. Figure 2 contains representative images observed of the three strains assayed. Biofilms established at 3, 6, 12, 24 or 48 h post-incubation at 37°C were used to assess the progression of the interaction between bacteria and the inert surfaces during the 1st hours of biofilm formation (Fig. 2A-D). Extracellular components which were likely to initiate attachment to the inert surface were observed within 3 h PI (Fig. 2E-F). This extracellular substance could be the exopolysaccharide or Extracellular Polymeric Substance (EPS) described as the main component of bacterial biofilms (7).

This material was observed in all preparations in large quantities by alcian blue staining, supporting its polysaccharide nature. It has been reported that the proportion of EPS in biofilms can comprise between 50-90% of the total organic material (Vu *et al*., 2009) and that the extracellular matrix plays a primary role in bacterial attachment to abiotic and cellular surfaces in the initial stages of biofilm formation (Costerton *et al*., 1987). The presence of a capsule in *Gallibacterium anatis* which could play a role in the extracellular matrix formation has been suggested previously (Bojesen *et al*., 2004) but remains to be fully described. After 3 h of incubation both the extracellular material (Fig. 2E) and microvesicles (Fig. 2F) presenting a similar structure and dimensions to those described previously in *Actinobacillus pleuropneumoniae* and *Avibacterium paragallinarum* (Negrete-Abascal *et al*., 2000; Ramon Rocha *et al*., 2006) were observed. Alongside EPS and different proteins, biofilms have been described as consisting of DNA and Outer Membrane Vesicles (OMV), a particular finding in Gram-negative bacteria. The role of OMV's was recently demonstrated by SEM on biofilms of *Helicobacter pylori* showing that OMV's enhances *H. pylori* biofilm formation (Yonezawa *et al*., 2009). In *Av. paragallinarum*, the study of OMV's indicated that they contained immunogenic proteins, proteases, putative RTX proteins, haemagglutinins and nucleic acids (Ramon Rocha *et al*., 2006). *Av. paragallinarum* OMV's agglutinated glutaraldehyde-fixed chicken red blood cells, thus indicating that they contained haemagglutinating antigens.

Some *G. anatis* strains are able to agglutinate avian or mammalian erythrocytes or both (Zepeda *et al*., 2009) but until now, there are no reports on OMV's from this bacterium. In addition, the ability to haemagglutinate erythrocytes correlates with *Staphyloccus epidermidis* adherence to plastic and intravenous catheters (Rupp and Archer, 1992). Consequently, haemaglutinating antigens and adhesins at the Gallibacterium surface could be taking part in biofilm formation however, the identification and characterization of those will have to be addressed in future studies. At 6 or 48 h post-incubation, filamentous-like structures joining different bacterial cells were observed (Fig. 2G-H).

A fraction of cells showed appendage-like structures forming bridges between them (Fig. 2B, D and G). Tubular structures and channels were also observed within biofilms of all three strains. These channels may permit oxygen and nutrients circulation or waste removal as has been described (Costerton *et al*., 1999; Sandal *et al*., 2007). Other structures which we were unable to identify were also observed (Fig. 2G). Structures such as cords and other filamentous components (Fig. 2H) were also observed at 24 and 48h biofilms. The cord-like structures observed in Gallibacterium biofilms were similar to those previously described from *Pseudomonas fluorescens* by Baum *et al*. (2009). Cell surface hydrophobicity, the presence of fimbriae and flagella, the composition and production of the Extracellular Polymeric Substance (EPS)

are all factors that strongly influence the rate and degree of attachment of microbial cells to different surfaces (Donlan, 2002). Different pathogenic members of the Pasteurellaceae family such as *Pasteurella* sp., Mannheimia haemolytica (Olson *et al*., 2002), *Actinobacillus pleuropneumoniae* (Kaplan and Mulks, 2005), *Hemophilus parasuis* (Jin *et al*., 2006), *A. actinomycetemcomitans* (Kaplan *et al*., 2003), *H. influenzae* (Webster *et al*., 2006) and *Histophilus somni* (Sandal *et al*., 2007) have the capability to form biofilms, in a similar way as we have shown in the present investigation on Gallibacterium. Pathogenic microorganisms associated with biofilm formation are currently a focus of intensive research due to their involvement in a large number of chronic infectious diseases. Biofilm formation is also believed to play an important role in infection immunity and protection against antimicrobial agents (Costerton *et al*., 1999).

CONCLUSION

The biofilm formation as demonstrated for Gallibacterium suggests that this mode of action could be important for the persistence of this organism on the surfaces of different tissues and possibly for its ability to cause infections beyond its natural habitat inside the host. However, these roles and the different specific components involved in these processes remain to be elucidated.

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REFERENCES

Addo, P.B. and K. Mohan, 1985. Atypical *Pasteurella haemolytica* type A from poultry. Avian Dis., 29: 214-217.

Baum, M.M., A. Kainovic, T. O'Keeffe, R. Pandita, K. McDonald, S. Wu and P. Webster, 2009. Characterization of structures in biofilms formed by a *Pseudomonas fluorescens* isolated from soil. BMC Microbiol., 9: 103-103.

Bisgaard, M., 1977. Incidence of *Pasteurella haemolytica* in the respiratory tract of apparently healthy chickens and chickens with infectious bronchitis. Characterisation of 213 strains. Avian Pathol., 6: 285-292.

Bojesen, A.M., O.L. Nielsen, J.P. Christensen and M. Bisgaard, 2004. *In vivo* studies of *Gallibacterium* anatis infection in chickens. Avian Pathol., 33: 145-152.

Bojesen, A.M., S.S. Nielsen and M. Bisgaard, 2003. Prevalence and transmission of haemolytic *Gallibacterium* species in chicken production systems with different biosecurity levels. Avian Pathol., 32: 503-510.

Christensen, H., M.B.A.M. Bojesen, R. Mutters and J.E. Olsen, 2003. Genetic relationships among avian isolates classified as *Pasteurella haemolytica*, *Actinobacillus salpingitidis* or *Pasteurella anatis* with proposal of Gallibacterium anatis gen. nov., comb. nov. and description of additional genomospecies within Gallibacterium gen. nov. Int. J. Syst. Evol. Microbiol., 53: 275-287.

Costerton, J.W., K.J. Cheng, G.G. Geesey, T.I. Ladd, J.C. Nickel, M. Dasgupta and T.J. Marrie, 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol., 41: 435-464.

Costerton, J.W., P.S. Stewart and E.P. Greenberg, 1999. Bacterial biofilms: A common cause of persistent infections. Science, 284: 1318-1322.

De Oliveira-Garcia, D., M. Dall'Agnol, M. Rosales, A.C.G.S. Azzuz, N. Alcantara, M.B. Martinez and J.A. Giron, 2003. Fimbriae and adherence of *Stenotrophomonas maltophilia* to epithelial cells and to abiotic surfaces. Cell. Microbiol., 5: 625-636.

Donlan, R.M., 2002. Biofilms: Microbial life on surfaces. Emerg. Infect. Dis., 8: 881-890.

Fey, P.D. and M.E. Olson, 2010. Current concepts in biofilm formation of *Staphylococcus epidermidis*. Future Microbiol., 5: 917-933.

Fredheim, E.G., C. Klingenberg, H. Rohde, S. Frankenberger, P. Gaustad, T. Flaegstad and J.E. Sollid, 2009. Biofilm formation by *Staphylococcus haemolyticus*. J. Clin. Microbiol., 47: 1172-1180.

Garcia-Gomez, E., S. Vaca, A. Perez-Mendez, J. Ibarra-Caballero, V. Perez-Marquez, V.R. Tenorio and E. Negrete-Abascal, 2005. *Gallibacterium anatis*-secreted metalloproteases degrade chicken IgG. Avian Pathol., 34: 426-429.

Jin, H., R. Zhou, M. Kang, R. Luo, X. Cai and H. Chen, 2006. Biofilm formation by field isolates and reference strains of *Haemophilus parasuis*. Vet. Microbiol., 118: 117-123.

Kaplan, J.B. and M.H. Mulks, 2005. Biofilm formation is prevalent among field isolates of *Actinobacillus pleuropneumoniae*. Vet. Microbiol., 108: 89-94.

Kaplan, J.B., M.F. Meyenhofer and D.H. Fine, 2003. Biofilm growth and detachment of *Actinobacillus actinomycetemcomitans*. J. Bacteriol., 185: 1399-1404.

Kristensen, B.M., D. Frees and A.M. Bojesen, 2010. GtxA from *Gallibacterium anatis*, a cytolytic RTX-toxin with a novel domain organisation. Vet. Res., 41: 25-25.

Mraz, O., P. Vladik and J. Bohacek, 1976. Actinobacilli in domestic fowl. Zentralbl. Bakteriol. Orig. A, 236: 294-307.

Mushin, R., Y. Weisman and N. Singer, 1980. *Pasteurella haemolytica* found in the respiratory tract of fowl. Avian Dis., 24: 162-168.

Negrete-Abascal, E., R.M. Garcia, M.E. Reyes, D. Godinez and M. de la Garza, 2000. Membrane vesicles released by *Actinobacillus pleuropneumoniae* contain proteases and Apx toxins. FEMS Microbiol. Lett., 191: 109-113.

Neubauer, C., M. de Souza-Pilz, A.M. Bojesen, M. Bisgaard and M. Hess, 2009. Tissue distribution of haemolytic *Gallibacterium anatis* isolates in laying birds with reproductive disorders. Avian Pathol., 38: 1-7.

Olson, M.E., H. Ceri, D.W. Morck, A.G. Buret and R.R. Read, 2002. Biofilm bacteria: Formation and comparative susceptibility to antibiotics. Can. J. Vet. Res., 66: 86-92.

O'toole, G.A. and R. Kolter, 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol., 30: 295-304.

Pompilio, A., R. Piccolomini, C. Picciani, D. D'Antonio, V. Savini and G. di Bonaventura, 2008. Factors associated with adherence to and biofilm formation on polystyrene by *Stenotrophomonas maltophilia*: The role of cell surface hydrophobicity and motility. FEMS Microbiol. Lett., 287: 41-47.

Ramirez, S., A. Zepeda, V. Vega, V. Morales, L. Perez, S. Diaz and V.E. Soriano, 2007. *In vitro* adhesion assays of *Gallibacterium* to chicken tracheal or oviductal epithelial cells. Electronic Proceedings of the 144th American Veterinary Medical Association-American Association Avian Pathology Washington, DC USA.

Ramon Rocha, M.O., O. Garcia-Gonzalez, A. Perez-Mendez, J. Ibarra-Caballero, V.M. Perez-Marquez, S. Vaca and E. Negrete-Abascal, 2006. Membrane vesicles released by *Avibacterium paragallinarum* contain putative virulence factors. FEMS Microbiol. Lett., 257: 63-68.

Rohde, H., C. Burdelski, K. Bartscht, M. Hussain, F. Buck *et al*., 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol. Microbiol., 55: 1883-1895.

Rupp, M.E. and G.L. Archer, 1992. Hemagglutination and adherence to plastic by *Staphylococcus epidermidis*. Infect. Immun., 60: 4322-4327.

Sandal, I., W. Hong, W.E. Swords and T.J. Inzana, 2007. Characterization and comparison of biofilm development by pathogenic and commensal isolates of *Histophilus somni*. J. Bacteriol., 189: 8179-8185.

Shaw, D.P., D.B. Cook, S.K. Maheswaran, C.J. Lindeman and D.A. Halvorson, 1990. *Pasteurella haemolytica* as a co-pathogen in pullets and laying hens. Avian Dis., 34: 1005-1008.

Vu, B., M. Chen, R.J. Crawford and E.P. Ivanova, 2009. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules, 14: 2535-2554.

Webster, P., S. Wu, G. Gomez, M. Apicella, A.G. Plaut and J.W. St Geme, 2006. Distribution of bacterial proteins in biofilms formed by non-typeable *Haemophilus influenzae*. J. Histochem. Cytochem., 54: 829-842.

Yonezawa, H., T. Osaki, S. Kurata, M. Fukuda and H. Kawakami *et al*., 2009. Outer membrane vesicles of *Helicobacter pylori* TK1402 are involved in biofilm formation. BMC Microbiol., 9: 197-197.

Zepeda, A., S. Ramirez, V. Vega, V. Morales and M. Talavera *et al*., 2009. Hemagglutinating activity of *Gallibacterium* strains. Avian Dis., 53: 115-118.