Short communication

Chicken erythrocyte Invasion Capability of a *Mycoplasma synoviae* Strain isolated in Argentina

Capacidad de invasión de los eritrocitos de pollo de una cepa de *Mycoplasma synoviae* aislada en Argentina

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Abstract: *Mycoplasma synoviae* is one of the most important pathogens in poultry industry and often causes diseases of a chronic and persistent nature. Little is known about the mechanisms of persistence in the host and the strains differ significantly in invasiveness and pathogenicity. A recent study demonstrated the capacity of *M. synoviae* to invade chicken erythrocytes (CER). The aim of this study was to determine whether a field *M. synoviae* strain isolated from an Argentinean laying hen farm has the same cell invasiveness capacity. The test used for this purpose was the gentamicin invasion assay. Samples of chicken erythrocytes *in vitro* infected with the *M. synoviae* field strain and control infective strains (*M. synoviae* WVU 1853 and *M. gallisepticum,* 14102) were taken 2, 4 and 24 hours after infection, inoculated in Frey broth with or without gentamicin, plated in Frey agar and incubated at 37 ºC for 4-6 days. *M. gallisepticum* 14102 treated with gentamicin grew well in the agar plates after 2 hours of infection. *M. synoviae* WVU 1853 and *M. synoviae* field strain treated with gentamicin grew in the agar plates after 24 hours of inoculation. It is very likely that the strains recovered from the infected chicken cells had invaded the erythrocytes and escaped the mycoplasmacidal effect of gentamicin. This study provides the first evidence of nonphagocytic cell invasion capability of a *M. synoviae* strain isolated in Argentina.

Keywords: *Mycoplasma synoviae*, cell invasion, chicken erythrocytes.

Resumen: *Mycoplasma synoviae* es uno de los patógenos más importantes en la industria de aves de corral y a menudo causa enfermedades de naturaleza crónica. Poco se sabe acerca de los mecanismos de persistencia en el hospedador y las cepas difieren significativamente en la invasividad y patogenicidad. Un estudio reciente demostró la capacidad de *M. synoviae* para invadir los eritrocitos de pollo (CER). El objetivo de este estudio fue determinar si una cepa de campo de *M. synoviae*, aislada de una granja de gallinas de postura de la Argentina, tiene la misma capacidad de invasión celular. La prueba utilizada para este fin fue el ensayo de invasión de gentamicina. Muestras de eritrocitos de pollo, infectadas *in vitro* con la cepa de campo de *M. synoviae* y con cepas de referencia como controles positivos (*M. synoviae* WVU 1853 y *M. gallisepticum* 14102), se tomaron 2, 4 y 24 horas después de la infección, se inocularon en caldo Frey con o sin gentamicina, se plaquearon sobre agar Frey y se incubaron a 37 ºC durante 4-6 días. La cepa de *M. gallisepticum*,14102 tratada con gentamicina desarrolló bien en las placas de agar 2 horas después de la inoculación. La cepa de *M. synoviae* WVU 1853 y la cepa de campo tratadas con gentamicina desarrollaron en las placas de agar después de 24 horas de la infección. Es muy probable que las cepas recuperadas de las células de pollo infectadas invadieran los eritrocitos y escaparan al efecto micoplasmacida de la gentamicina. Este estudio proporciona la primera evidencia de la capacidad de una cepa *de M. synoviae* aislada en la Argentina para invadir células no fagocíticas.

Palabras clave: *Mycoplasma synoviae*, invasión celular, eritrocitos de pollo.

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Introduction

Mycoplasma synoviae is one of the most important pathogens in poultry worldwide. Infection may produce a disease that ranges from subclinical to severe. Clinical manifestations vary depending on the strain, and some appear to have a greater tropism for the synovial membranes or the respiratory tract than others (Kleven *et al*, 1975). Adhesion of mycoplasma to epithelial cells by specific cytodhesins is considered to be the first step in pathogenesis and has been mainly studied in relation to the respiratory tract epithelium (Bencina D., 2002; Narat *et al*., 1998). In a recent study (May *et al*, 2007), the sialidase activity of different *M. synoviae* strains has been evaluated as a virulence factor associated to strain invasiveness of the host. In agreement with its pathogenic profile, two *M. synoviae* strains isolated from chicken farms in Argentina had high sialidase activity (Cerdá *et al*, 1998).

Evasion from the immune system by changing surface proteins has been studied in *M. synoviae* as well as in many other *Mycoplasma* species as a mechanism of persistence in the host (Noormohammadi *et al*., 2000; Winner *et al*., 2003). More recently, nonphagocytic cell invasion capability has been found in *M. gallisepticum* and *M. synoviae* as another way of host defense evasion and persistence in the host (Much *et al*., 2002; Dusanic *et al*., 2009). The aim of the present study was to determine if a *M. synoviae* strain isolated in Argentina from a laying hen farm, in which hens were severely affected with clinical signs of synovitis, have comparatively the same capacity to invade chicken erythrocytes as the one of a reference *M. synoviae* strain.

Matherials and Methods

Mycoplasma cultures

M. synoviae reference strain WVU 1853, *M. gallisepticu*m strain 14102 and a *M. synoviae* strain JR2010 isolated in Argentina from a laying hen farm located in Buenos Aires province were used. Prior to the cell infection, mycoplasma cultures were grown at 37 ºC in Frey´s broth until reaching a mid-exponential phase, as indicated by the metabolic color change of the medium (phenol red). The number of viable mycoplasmas in the suspension was determined by plating ten fold serial dilutions on Frey´s agar plates, followed by incubation at 37 ºC for 4 to 6 days.

Chicken red blood cells

Blood samples were taken from adult specific pathogen-free chickens and mixed with EDTA. Chicken erythrocytes (CER) were separated from the collected blood by adding sterile phosphate buffer saline (PBS) pH 7.4 and centrifugation at 400 g for 10 min. CER were washed 3 times in PBS and were re-suspended in PBS to a final concentration ranging from 5 x 10^5 to 10^6 cells/ml.

Determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of gentamicin

The susceptibility of the mycoplasma strains to gentamicin was determined by inoculating 25 µl samples of each mycoplasma cultures (10^5 to 10^6 CFU/sample) into 100 µl of Frey's broth containing two fold serial dilutions of a gentamicin solution into a u-bottom 96 wells plate. After 24 hours of incubation at 37 °C, the MIC was calculated as the minimum dilution of antibiotic that avoid the culture to grow. To determine the MBC of gentamicin for each strain, 10 µl of each non growing dilution of the broth was inoculated in agar plates without gentamicin and the plates were incubated at 37 ºC for 4 to 6 days.

Experimental infection of chicken erythrocytes (CER)

The mycoplasma strains used for the infections were incubated until they reached the late logarithmic phase of growth 100 µl of broth containing 10^7 to 10^8 CFU were used to inoculate 1 ml (5-6 x 10^5 cells) of CER samples suspended in Frey's broth. The multiplicity of infection (MOI) ranged from 10 to 100 mycoplasmas per chicken cell. CER infected with mycoplasma and uninfected samples of CER were used as negative controls. All CER samples were incubated at 37-38 ºC.

Gentamicin invasion assay

The presence of intracellular mycoplasmas inside chicken erythrocytes was determined using the gentamicin invasion assay described by Winner *et al* (2000) and Vogl *et al* (2008). This assay is based on the inability of gentamacin to cross eukaryotic cell membranes exerting its antibiotic activity only against extracellular mycoplasmas. Briefly, 2-4 and 24 hours after infection, the content of each selected well was centrifugated for 10 min at 300 g. Then the pellets were re-suspended either in 1 ml of Frey's broth without gentamicin, or in 1 ml of Frey's broth supplemented with 1,000 µg gentamicin (concentration more than 4 times up the MBC). Cells were re-suspended several times during the 4 h of incubation at 37 ºC. After treatment, the cell suspensions were washed three times with PBS pH 7.4. The infected cells treated with gentamicin were re-suspended in approximately 150 µl of Frey's broth and 20 µl of samples were plated

onto Frey's agar and incubated at 37 ºC. The infected cells, which were not treated with gentamicin were re-suspended in 1.5 ml of Frey's broth and plated as described above. Uninfected cells were incubated in Frey's broth in the same way as the infected cells and were used as negative controls. After 4-6 days, mycoplasma colonies on the plates were identified and counted.

Results and Discussion

In agreement with previous reports, *M. gallisepticum* strain 14102 was found viable into the erythrocytes treated with gentamicin after two hours of infection (Vogl *et al*, 2008) (Figure 1). However, *M. synoviae* WVU 1853 (Figure 2) was viable in erythrocytes only after 24 hours of infection (Figure 3), much

Table 1. Results of the cell infection with different mycoplasma strains.

Strains	MBC (µg/ml)	Growth 2hours PI		Growth 4 hours PI		Growth 24 hours PI	
		CER	CER + Gen	CER	CER + Gen	CER	CER + Gen
M. synoviae wvu 1853	125	+	-	+	-	+	+
M. synoviae JR2010	125	+	-	+	-	+	+
M. gallisepticum 14102	250	+	+	+	+	+	+

PI: Post Infection; CER: Chicken Erythrocytes: GEN: Gentamicin; MBC: Minimal Bactericidal Concentration for gentamicin.

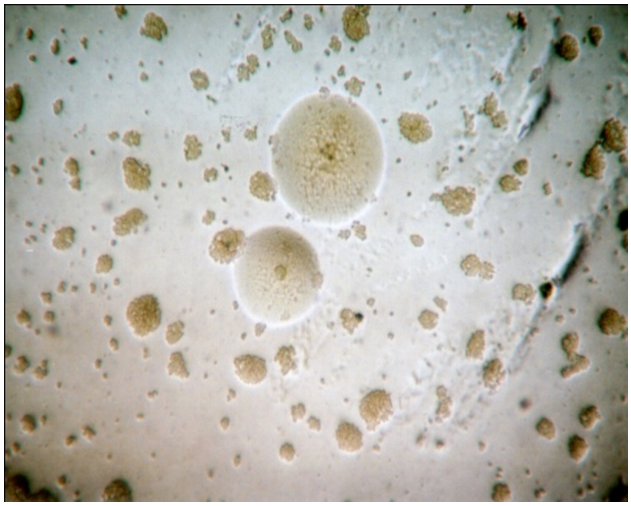

Figure 1. M. gallisepticum colonies 2 hours PI (CER+GEN).

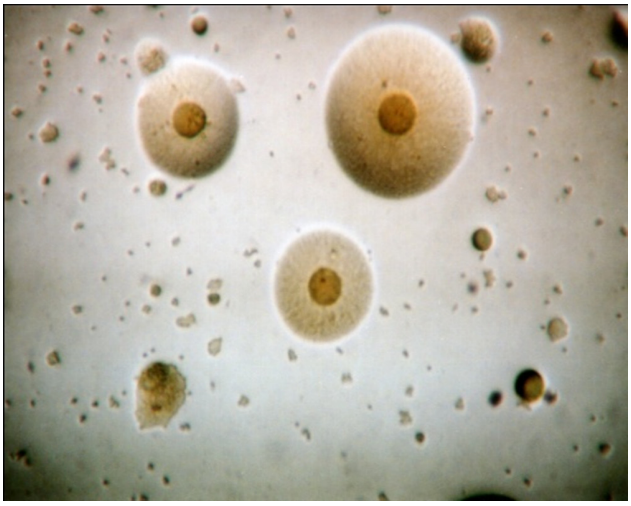

Figure 2. M. synoviae wvu 1853 colonies 4 hours PI (CER).

Figure 3. M. synoviae wvu 1853 colonies 24 hours PI (CER+GEN).

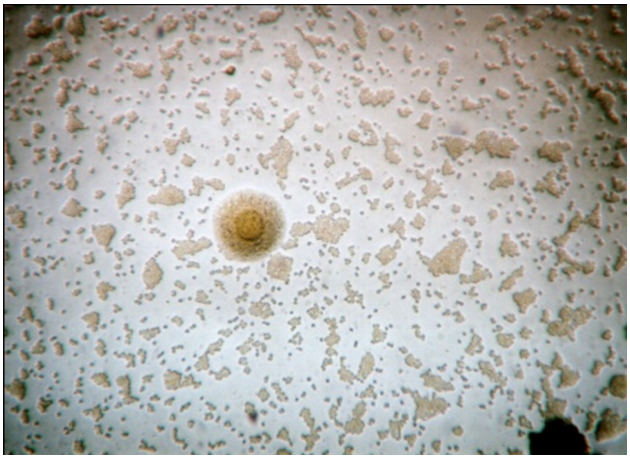

Figure 4. M. synoviae field strain colonies 24 hours PI (CER+GEN).

later than the lapse of time observed by Dusanic *et al* (3 hours after infection) (Table 1). These differences could be explained by the number of laboratory passages to which reference strains were subjected. The *M. synoviae* JR2010 field strain showed the same invasiveness behavior as the reference strain (Figure 4). Considering the fact that in the present study more than 4 times of the usual gentamicin concentration was used in the MBC test to kill the extracellular mycoplasma cells, it is very likely that the *M. gallisepticum* and *M. synoviae* organisms recovered from the infected chicken cells had invaded the erythrocytes and escaped from the mycoplasmacidal effect of the antibiotic. The results provided in this study are consistent with the data published on the ability of *M. synoviae* to *in vitro* invade CER (Dusanic *et al*., 2009), and is the first report on this subject in a strain isolated in Argentina. Further studies will be conducted to evaluate *in vivo* this invasiveness behavior with other field isolates as well as to study the antibiotic effectiveness in infected CER.

Conclusions

This *in vitro* study provides the first evidence that a *M. synoviae* strain isolated in Argentina is able to penetrate inside chicken erythrocytes. This finding, together with the sialidase activity described before (Cerdá *et al*, 1998) on strains isolated from the same country, may contribute to understand the virulence of this mycoplasma species in the region. The advantage of entering into erythrocytes might be not only a mechanism to reach new sites of infection, but also to escape from the immune system of the host and avoid the contact with specific antibiotics.

Conflict of interest

All authors declare that there are not conflict of interests, including financial, personal or other relationships with other people or organizations that could inappropriately influence the work.

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