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Low temperature and dust favour *in vitro* survival of *Mycoplasma hyopneumoniae*: time to revisit indirect transmission in pig housing

Christopher Browne*, Anette Loeffler, Hannah Holt, Yu-Mei Chang, David H Lloyd, Amanda Nevel.

The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, AL9 7TA, UK

+44 1707 666 631

Survival of *Mycoplasma hyopneumoniae*

* Corresponding author: Tel. +44 1707 666 631

E-mail address: cbrowne@rvc.ac.uk (C. Browne).

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Significance and impact of the study

Understanding the transmission of *M. hyopneumoniae* and optimising biosecurity practices is key to reducing the use of antimicrobial agents to control this pathogen. Direct transmission of the pathogen between pigs is the main route of spread and its lack of cell wall may compromise its resilience outside the host. The results from our study show that *M. hyopneumoniae* can survive for up to several days on dry surfaces and therefore may have the potential to infect pigs by indirect transmission. Factors influencing survival of *M. hyopneumoniae* outside the host are further elucidated.

Abstract

Porcine enzootic pneumonia (EP) caused by *Mycoplasma hyopneumoniae* adversely affects pig welfare and is associated with major economic losses in the pig industry worldwide. Transmission is predominantly by direct contact but the role of indirect transmission remains poorly understood. This study examined survival of six *M. hyopneumoniae* isolates dried onto five different surfaces encountered in pig units, and exposed to temperatures of 4°C, 25°C and 37°C for up to 12 days. Survival of the organisms was determined by recovering the organism from the surface material and culturing in Friis broth. Data was analysed by logistic regression to identify factors influencing survival of *M. hyopneumoniae*. Maximum survival was eight days for all isolates on at least one surface (except stainless steel) at 4°C and was limited to two days at 25°C and 37°C. Overall, dust and polypropylene copolymer supported *M. hyopneumoniae* survival the longest when compared with other surface materials. In conclusion, we have demonstrated that *M. hyopneumoniae* can survive outside the host for at least eight days.

Key words

Dust, Survival, Indirect transmission, Management, Mycoplasma, Temperature

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Introduction

Porcine enzootic pneumonia (EP) caused by *Mycoplasma hyopneumoniae* is associated with major economic losses in the pig industry worldwide (Maes *et al*. 1996). Vaccination programmes are generally cost-effective and commonly used. However, they do not prevent transmission (Villarreal *et al*. 2011) or colonisation of the organism (Meyns *et al*., 2006). Management practices optimising the pigs' environment are key for controlling the disease (Kobish and Friis 1996). Further, strategic antimicrobial therapy can contribute to control of the organism, either alone or in combination with vaccination (Maes *et al*. 2008). However, caution should be taken given increasing concerns over the development of antimicrobial resistance in *M. hyopneumoniae* isolates (Vicca et al., 2004).

Transmission of *M. hyopneumoniae* is primarily by direct contact between pigs but there is good evidence that spread via aerosol also plays an important role (Fano *et al*., 2005) with airborne transport of *M. hyopneumoniae* recorded at 9.2km (Otake *et al*., 2010). Reinfections in farms which appear to have eliminated the organism are common (Goodwin 1985) and other routes of transmission are likely, for example contaminated slaughterhouses and transport vehicles have been implicated in a Swiss study (Hege *et al.* 2002). However, indirect transmission via environmental surfaces remains poorly understood. Eradication strategies, based on vaccination, air filtration, antimicrobial therapy and movement restrictions, have shown some success in the past in geographically confined regions such as Finland and Switzerland (Rautiainen *et al.* 2001; Stärk *et al*. 2007) and the United States (Holst et al., 2015). Advances in understanding the epidemiology of *M. hyopneumoniae* are hampered by the difficulties associated with culturing the organism (Kobisch and Friis 1996). Molecular methods have helped in recent years to demonstrate contamination of surfaces, but not viability of bacteria (Stärk *et al.* 1998; Marois *et al.* 2008).

The survival of *M. hyopneumoniae* outside the host was first explored *in vitro* by Friis (1973) and subsequently by Goodwin (1985) who demonstrated survival of organisms when dried at room temperature on coverslips for up to four days and in open bijou tubes for up to seven days, respectively. Their results suggest that *M. hyopneumoniae* can survive in the environment and

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thereby act as a potential source for indirect transmission. However, *in vivo* studies on experimental indirect transmission reported by Goodwin in 1972 (Goodwin 1972) and recently, Batista *et al*., (2004) have failed to cause infection. Understanding the survival outside the host is imperative to implement appropriate control strategies. This study determines the duration of survival of six different *M. hyopneumoniae* isolates on five surfaces commonly encountered in pig units at three temperatures.

Results and discussion

All six *M. hyopneumoniae* isolates survived for at least one day at 4°C on all five surfaces with the longest survival of eight days seen with at least one isolate on four surfaces (Table 1). This is twice as long as the four-day survival demonstrated at room temperature by Friis (1973). On stainless steel, maximum survival was two days when incubated at 4°C. Similarly, survival of all strains was reduced to a maximum of two days when incubated at 25°C or 37°C, irrespective of surface material (Table 1). No *M. hyopneumoniae* strain survived for more than one day when incubated on PC at either 25°C or 37°C and none survived incubation on GPP at 37°C.

The survival of *M. hyopneumoniae* on dust is of particular interest. Dust can affect health by causing irritation to the respiratory tract (Donham and Leininger 1984) and provide a vector for pathogenic organisms to create disease (Curtis *et al*., 1975). Indeed, dust has frequently been associated with increased risk of respiratory disease in pigs (Underdahl *et al.* 1982; Donham 1991) but whether this occurs through impairment of the respiratory epithelium or by allowing pathogens to survive longer, or a combination of both, is not known. Increased levels of dust in the environment of pigs are associated with poor hygiene and management practices, for example where stocking density is high or ventilation is poor this has been shown to exacerbate respiratory disease (Stärk 1999; 2000). However, the role of dust in the transmission of *M. hyopneumoniae* is not clearly understood; Stärk *et al.* (1998) failed to identify *M. hyopneumoniae* using nested PCR in dust samples from pig farms with acute respiratory problems but suggested that inhibitors found in dust may have had a negative effect on the PCR assay.

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Irrespective of temperature and surface material, field isolates FI9, FI7, FI5, FI1 and MhJ had decreased odds of survival, compared with Mh232 (Table 2). Irrespective of surface material and isolate type, temperatures of 37ºC and 25ºC decreased the odds of *M. hyopneumoniae* surviving when compared with survival at 4ºC (OR: 0.06, $P < 0.001$ and 0.15, $P < 0.001$ respectively). *M. hyopneumoniae* had higher odds of survival on all the materials tested compared with survival on glass, although this was less evident for stainless steel (OR: 1.49, $P = 0.06$) (Table 2).

The lack of a cell wall in *Mycoplasma* species (Razin 1969) renders them susceptible to osmotic (Razin 1963) and heat (Friis 1974) shock and, in our study, temperature had the largest influence on *M. hyopneumoniae* survival duration. However, the mechanisms by which this occurs are not fully understood. The small genome size of *M. hyopneumoniae* (Minion *et al.* 2004) limits its capabilities of nutrient utilisation which manifests as strict host preferences (Vasconcelos *et al.* 2005) and culture *in vitro* requires a highly specialised growth medium. In accordance with our study, other *Mycoplasma* species have also been shown to survive longer when exposed to cooler temperatures. Nagatomo *et al.* (2001) found similar survival patterns with *M. gallisepticum* on paper discs, showing that survival was longest at 4°C, but significantly reduced at higher temperatures (30°C and 37°C). Some bacteria species, for example *Psychrobacter cryopegella* were able to metabolise and grow at -10°C. Under these cold temperatures, bacteria can adapt by altering their wall structure, become smaller in size and had more outer membrane vesicles (Bakermans *et al*., 2003).

Indirect transmission has been demonstrated with other *Mycoplasma* species including *M. synoviae* (Marois *et al.* 2005) and *M. gallisepticum* (Dhondt *et al.* 2007) but, as indicated previously, Goodwin (1972) was unable to indirectly infect pigs with *M. hyopneumoniae*. Furthermore, although the organism has been detected in the airspace of pig units Stärk *et al.* (1998) and in scald tanks from slaughterhouses (Marois *et al.* 2008) using PCR and there are no reports of detecting viable *M. hyopneumoniae* organisms outside of the host. Whether the lack of identification of viable organisms was because they were not present or because of the difficulties of culturing the organism is not known (Kobisch and Friis 1996).

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Our findings confirm that *M. hyopneumoniae* can survive outside the host for at least eight days at 4°C and this may provide an opportunity for indirect transmission. However, our study was conducted in a laboratory under controlled conditions and may not reflect the situation on farm. For example, sunlight, low level of nutrients, variable pH shifts, moisture, toxins or predation occur in the natural environment and may prevent survival (Winfield and Groisman 2003). Further work should explore the survival of *M. hyopneumoniae* in the field to determine whether the organism survives under these conditions and to determine the infection dose required for indirect transmission. McAuliffe *et al.* (2006) demonstrated that some *Mycoplasma* species were able to form a biofilm under stressful conditions when outside the host. Indeed, when *M. bovis* biofilms were subjected to a temperature of 50°C, survival rates were higher than planktonic cells after 40 min. Furthermore, *M. bovis* biofilms were able to survive eight h longer than planktonic cells once dried. It is worth noting that not all *Mycoplasma* species have been shown to form biofilms, and the evidence that *M. hyopneumoniae* is capable of this is limited (Raymond *et al*., 2015).

Our results, showing *M. hyopneumoniae* survival outside the host for at least eight days at cooler temperatures, shed new light on the potential for indirect transmission routes and provide a possible explanation for previous EP elimination failures. Indeed, in clinical situations, elimination of *M. hyopneumoniae* from pig units is more successful when previously infected premises are left vacant for a period of several weeks before the reintroduction of *M. hyopneumoniae* free pigs (Stärk *et al.* 2007). Further, cleaning and disinfecting between batches of pigs disrupts the infection chain and therefore can help prevent *M. hyopneumoniae* infection (Maes *et al*., 1996). Studies on indirect transmission are now needed to further explain the mechanisms for *M. hyopneumoniae* survival outside their hosts and thereby transmission under field conditions. This can inform the design of hygiene strategies and may contribute to the success of eradication programmes, ultimately reducing the reliance on antibiotic use on farm.

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Materials and method

Bacterial strains and culture

Four field isolates (FI9, FI7, FI5, FI1), collected from EP-like lesions of porcine lung tissue at a UK abattoir and confirmed by 649-bp fragment PCR of the 16S rRNA gene (Stakenborg *et al.* 2006), and two reference strains, *M. hyopneumoniae* J (MhJ) (NCTC10110) and *M. hyopneumoniae* 232 (Mh232) (AE017332.1) , were included. All strains were grown in liquid Friis medium as described by Kobish and Friis (1996) and incubated at 37°C until growth was evident by a colour change using the pH dependent indicator, phenol red (red to yellow). Growth was quantified using ten-fold serial dilutions in Friis medium. The highest dilution showing a colour change was assumed to contain one colour-changing unit (CCU). The number of *M. hyopneumoniae* organisms in a suspension is expressed as CCU/ 0.2 ml (Taylor-Robinson and Furr 1981).

Experimental design

Five different test surface materials were used: glass (Fisher Scientific, Loughborough, UK), general purpose polystyrene (GPP; Sterilin, Newport, UK) (GPP supplied as sterile 6-well plates), dust (100 mg) collected from a Royal Veterinary College pig research facility air duct and deposited on GPP, stainless steel (SS; G.E. Baker Ltd, Suffolk, UK) and polypropylene copolymer (PC; Paneltim®) (G.E. Baker Ltd, Suffolk, UK). Each test surface was autoclaved at 121°C for 10 min except the GPP which was sterilised by gamma irradiation. Each test surface was then placed into a sterile 6-well plate and seeded with 40µl of each culture grown to CCU 1×10^{10}. Culture suspensions were spread over an area of 1 cm^2 on each of the test materials and allowed to dry for 1.5 h at room temperature before incubation at either 4°C, 25°C or 37°C.

Recovery of dried *M. hyopneumoniae* from surfaces materials

M. hyopneumoniae were recovered immediately after drying (0), and after 1, 2, 4, 8 and 12 days, by adding 360 µl of Friis medium onto the surface of the test material, and mixing for five minutes on an orbital shaker (200 rotations/minute). Twenty microliters of the initial suspension from each test

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surface material was used to perform serial 10-fold dilutions (20 µl into 180 µl) into 96-well plates (Greiner bio-one GmbH, Frickenhausen, Germany), which were then incubated at 37°C for 14 days. Survival of *M. hyopneumoniae* strains was assessed in CCU. All experiments were carried out in duplicate.

Data analysis

The survival of *M. hyopneumoniae* is expressed as maximum duration in days, with mean number of CCU. A logistic regression model was used to identify factors influencing survival of *M. hyopneumoniae*. The outcome variable was survival (with CCU > 0 indicating survival) and predictors were strain, surface material and temperature. Time (in days) was included in the model as a covariate to control for time-dependency of survival. Initially all predictors were included in the model and retained if they were associated with survival with a $P \leq 0.05$. In the model the Mh232 strain, 4°C and glass was selected as the reference categories in order to compare the survival of the chosen variables. Analyses were performed in IBM SPSS Statistics for Windows, version 20.0 (Armonk, New York, USA, 2011).

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Conflict of interest statement

No conflict of interest declared.

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Table 1 Maximum survival duration of *M. hyopneumoniae* in days on different surfaces and at different temperatures with mean CCU.

Material	Temp	Mh232	FI9	FI7	FI5	FI1	MhJ
Glass	4 ºC	2 (1 x 10^{2})	1 (7 x 10^{2})	8 (3 x 10^{6})	8 (1 x 10^{0})	8 (1 x 10^{0})	4 (5 x 10^{2})
GPP		8 (2 x 10^{4})	2 (3 x 10^{1})	2 (5 x 10^{0})	1 (1 x 10^{2})	2 (3 x 10^{0})	4 (5 x 10^{2})
Dust		4 (1 x 10^{4})	8 (2 x 10^{4})	8 (3 x 10^{4})	2 (2 x 10^{2})	8 (3 x 10^{0})	4 (2 x 10^{6})
SS		2 (3 x 10^{4})	1 (7 x 10^{4})	1 (3 x 10^{4})	1 (5 x 10^{2})	1 (3 x 10^{5})	2 (3 x 10^{2})
PC		8 (2 x 10^{6})	1 (2 x 10^{5})	8 (3 x 10^{4})	1 (2 x 10^{4})	8 (5 x 10^{0})	8 (3 x 10^{1})
Glass	25 ºC	1 (5 x 10^{2})	2 (3 x 10^{0})	2 (5 x 10^{0})	0 (2 x 10^{5})	0 (2 x 10^{5})	0 (2 x 10^{8})
GPP		2 (3 x 10^{3})	0 (1 x 10^{3})	1 (3 x 10^{0})	1 (7 x 10^{0})	1 (5 x 10^{0})	0 (7 x 103)
Dust		1 (2 x 10^{2})	1 (5 x 10^{1})	2 (3 x 10^{0})	0 (2 x 10^{5})	2 (5 x 10^{0})	2 (5 x 10^{0})
SS		1 (5 x 10^{1})	1 (3 x 10^{1})	1 (7 x 10^{1})	1 (5 x 10^{0})	1 (7 x 10^{0})	2 (3 x 10^{0})
PC		1 (1 x 10^{2})	1 (5 x 10^{0})	1 (3 x 10^{1})	1 (5 x 10^{0})	1 (5 x 10^{1})	1 (5 x 10^{3})
Glass	37 ºC	1 (8 x 10^{9})	0 (3 x 10^{3})	0 (5 x 10^{5})	0 (2 x 10^{5})	0 (2 x 10^{5})	0 (5 x 10^{6})
GPP		0 (3 x 10^{5})	0 (1 x 10^{3})	0 (7 x 10^{3})	0 (2 x 10^{2})	0 (3 x 10^{6})	0 (2 x 10^{4})
Dust		2 (2 x 10^{2})	0 (2 x 10^{5})	0 (3 x 10^{9})	0 (2 x 10^{5})	0 (5 x 10^{2})	0 (2 x 10^{5})
SS		0 (5 x 10^{0})	0 (5 x 10^{6})	1 (5 x 10^{0})	0 (3 x 10^{6})	0 (5 x 10^{4})	1 (3 x 10^{2})
PC		1 (5 x 10^{0})	1 (3 x 10^{0})	0 (2 x 10^{5})	0 (3 x 10^{6})	0 (3 x 10^{9})	1 (2 x 10^{3})

Temp, temperature; CCU, colour changing units; SEM, standard error of the mean;

FI, field isolate; Mh232, *M. hyopneumoniae* 232; MhJ, *M. hyopneumoniae* J; GPP, general purpose polystyrene; SS, stainless steel; PC, polypropylene copolymer.

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Table 2 Multiple logistic regression analysis of the survival of *M. hyopneumoniae* strains on different surfaces materials and temperatures.

Parameter	Odds ratio [*]	95% Confidence Interval		*P*-value
		Lower	Upper	
Mh232 (reference)	1 [†]	-	-	-
FI9	0.28	0.17	0.45	<0.001
FI7	0.41	0.25	0.65	<0.001
FI5	0.16	0.09	0.26	<0.001
FI1	0.23	0.14	0.37	<0.001
MhJ	0.51	0.32	0.81	0.005
4 ºC (reference)	1 [†]	-	-	-
25 ºC	0.15	0.11	0.22	<0.001
37 ºC	0.06	0.04	0.10	<0.001
Glass (reference)	1 [†]	-	-	-
GPP	1.63	1.06	2.49	0.024
Dust	2.89	1.88	4.45	<0.001
SS	1.49	0.97	2.28	0.064
PC	1.96	1.28	3.00	0.002

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[*] Odds ratio (OR) of > 1 indicates increased odds of *M. hyopneumoniae* survival.

[†] Reference category; the group to which all the other categories in the variable are compared

Mh232, *M. hyopneumoniae* 232; FI, field isolate; MhJ, *M. hyopneumoniae* J; GPP, general purpose polystyrene; SS, stainless steel; PC, polypropylene copolymer.

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