Extended survival times of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* on kanekalon synthetic hair fibres

Celia Abolnik*[1] and Johan Gouws†

*Poultry Section, Department of Production Animal Studies, and †Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa

ABSTRACT The survival times of *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms) on washed and unwashed natural and synthetic kanekalon hair samples over a 5-d period were evaluated using the color changing unit method for comparison with results of previous studies conducted on natural hair. Regardless of whether synthetic or natural hair samples prewashed with a disinfectant shampoo were spiked with Mg or Ms, all viable organisms rapidly dropped below a count of 1×10^1/mL of culture. Unwashed natural hair seeded with a titer of approximately 1×10^6/mL of viable Mg or Ms decreased to 6×10^5/mL and 6×10^3/mL, respectively, by 4 h postseeding, but no viable Mg or Ms were detected on natural hair from 8 h onwards. By contrast, the titers of Mg and Ms on synthetic hair did not decline from the initial 1×10^6/mL seed dose up to 96 h postseeding, and, in fact, viable Mg and Ms was still detectable at 9 d postinfection. Application of a real-time quantitative single-tube duplex PCR assay confirmed that no proliferation of Mg or Ms had occurred on the synthetic hair samples, the cells simply remained viable. The unexpected finding that Mg and Ms survive for extended periods on synthetic kanekalon hair fibers raises the question of whether attachment to a surface is a prerequisite for the survival and persistence of Mg and Ms in the extra-host environment. Future studies should be aimed at determining whether other synthetic hair types or indeed other types of plastics commonly found in the poultry house offer similar survival advantages to mycoplasmas.

Key words: mycoplasma, synthetic, hair, survival, PCR

INTRODUCTION

The widespread avian respiratory pathogens *Mycoplasma gallisepticum* (**Mg**) and *Mycoplasma synoviae* (**Ms**) are, as all *Mollicutes*, self-replicating prokaryotes capable of survival outside of a host for extended periods, depending on the strain (Nagatomo et al., 2001; Bradbury, 2005). Numerous studies on the survival of mycoplasmas in the environment have been conducted (reviewed by Christensen et al., 1994), including 2 studies that investigated the survival of poultry mycoplasmas on natural hair. Some strains of Mg survived for up to 3 d on natural hair, but a strain of Ms survived only 8 h postinoculation. *Mycoplasma iowae* strains survived beyond 5 d on hair in the same experiment (Christensen et al., 1994).

In South Africa, many of the large poultry producers have banned the wearing of synthetic hair braids by poultry workers for biosecurity reasons. Synthetic hair extensions, a global cosmetic trend across many ethnic groups, are typically manufactured from kanekalon, toyokalon, or monofiber fibers. Kanekalon is composed of a combination of 2 monomers, acrylonitrile and vinyl chloride, and is a light, long-lasting synthetic fiber that withstands repeated washings and low-temperature heat styling. Toyokalon is composed of polyvinyl chloride, a softer synthetic hair fiber that tangles less, but lacks the natural look and color of kanekalon fiber. Monofiber is the highest quality synthetic hair, composed of acrylic (http://www.yale.edu/ynhti/national-curriculum/units/2011/5/11.05.10.x.html).

No mycoplasma survival studies have previously been published for synthetic hair. Our objectives were to determine whether synthetic hair provides a significant advantage (or disadvantage) over natural hair for the viability of Mg and Ms. Our original hypothesis was that no significant difference would be observed between the survival of Mg and Ms between unwashed natural and synthetic hair; therefore, we also compared the effects of treating both hair types with a brand of disinfectant shampoo that is commonly used in poultry houses. Survival times of Mg and Ms on washed and unwashed natural and synthetic hair samples was eval-

[1]Correspondingauthor: celia.abolnik@up.ac.za

uated using the color changing unit (**CCU**) method over a 5-d period to enable us to compare our results with those of previous survival studies of Mg and Ms in the environment (Christensen et al., 1994; Nagatomo et al., 2001).

MATERIALS AND METHODS

Preparation of Hair Samples

Natural human hair obtained from a local hairdressing salon was thoroughly and repeatedly rinsed in distilled water to remove any residual traces of shampoo and colorant. Synthetic kanekalon fiber hair samples (Premium X-pression, 1/350, Linda Manufacturing Company Ltd., Lagos, Nigeria) were purchased from a local supplier. Both types of hair were cut into sections weighing roughly 0.75 g each, folded into a loop, and secured with and elastic band. Both hair types were then rinsed numerous times in distilled water, followed by a 5% acetic acid wash to remove residual hair products, and a final rinse in distilled water. Hair samples were allowed to dry on sterile filter paper at room temperature. Natural and synthetic hair in sealed 1-L cell culture flasks (Nunc, Sep Sci, Honeydew, South Africa) were sterilized by irradiation treatment at a dose of 23.5 kilogray units [Synergy Sterilization SA (Pty) Ltd., Kempton Park, South Africa].

To avoid mechanical displacement of organisms by washing, the sterile hair samples were washed before the experiment. The F10 Hand Scrub (Health and Hygiene, Florida Hills, South Africa), is a common disinfectant used as a shampoo and body wash in the poultry industry. This product contains quaternary ammonium and biguanidine compounds (0.35%), nontoxic surfactants, sequestrants, and glycine. Twenty natural and synthetic hair samples each were removed under aseptic conditions and immersed in undiluted F10. Samples were rinsed in distilled water until most, but not all, of the soap had been removed, as evidenced by the presence of soap bubbles. Washed samples were dried on sterile filter paper in a laminar flow cabinet.

Strains, Propagation, and Titration

Mycoplasma gallisepticum strain NCIC10115 and *M. synoviae* strain ATCC25204 were propagated in mycoplasma broth (**MB**). The seed stock was aliquotted and frozen at −70°C until use. Aliquots of each Mg and Ms were defrosted and counting of the number of viable mycoplasmas per 1 mL was carried out using the CCU method of Taylor Robinson (1983). A trial run was conducted to ensure the correct dilutions were to be used during the experiment.

Spiking of Hair Samples

Hair samples were placed in 15-mL universal tubes, to which a 10 mL volume of 1×10^6/mL of Mg or Ms suspension in MB was added, with a negative control for each sample (treated or untreated natural or synthetic hair with plain MB). After a short vortexing step (15 s) to mix samples, hair samples were removed, drained of excess fluid on sterile filter paper, left to air dry in a laminar flow cabinet, and placed in clean bottles. Samples were incubated at ambient temperature for the duration of the experiment

Materials seeded with Mg and Ms were examined for viable organisms at the start of the experiment (within an hour) and then at 2, 4, 6, 12, and 24 h, and on d 2, 3, 4, and 5. For culture, 2 mL of MB was added to the sample and agitated on a vortex mixer for 20 s, then 20 μl of broth was added to 180 μL of MB and titrated for CCU in 5-fold. The remaining medium was immediately frozen at −80°C for later PCR analysis. Titrations were read for end-points after 5 d of incubation at 37°C.

DNA Extraction

One milliliter each of *M. gallisepticum* strain NCTC10115 (1.5×10^7/mL) and *M. synoviae* strain ATCC25204 (1.5×10^{10}/mL) cultures were harvested by centrifugation at 12,500 × *g* for 10 min at ambient temperature and extracted according to the recommended procedure for gram-negative bacteria using a PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, CA). Ten-fold serial dilutions of the DNA of Mg and Ms strains were prepared in the kit elution buffer for standard curves.

Quantitative Real-Time Duplex PCR

The method described by Sprygin et al. (2010) was modified by labeling probe MGmgc2 with MGB-FAM and probe MSvlhA with MGB-VIC. Primers and probes were synthesized by Life Technologies (Johannesburg, South Africa). Both sets of probes and primers were combined into a single mix consisting of 5 μL of PCR-grade water, 5 μL of VetMax quantitative PCR Master Mix (Life Technologies, Austin, TX), 0.5 μL of each of the 4 primers (Mg and Ms) at 12.5 pmol/μL, and 0.15 μL of each probe at 5 pmol/μL. Twelve microliters were aliquotted into each well of a 96-well thermal plate. Three microliters of DNA (standards in triplicate, samples in triplicate and controls) or water (negative controls) were added to the respective well. quantitative real-time duplex PCR (**qPCR**) was performed on a Step One Plus real-time thermocycler (Life Technologies) programmed at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 50°C for 45 s (data capture point).

RESULTS AND DISCUSSION

Mycoplasma Survival on Washed Hair

Regardless of whether washed synthetic or natural hair samples were spiked with Mg or Ms, all viable

Table 1. Survival of mycoplasmas on hair per milliliter of culture as assessed by the color changing unit method

	Mycoplasma gallisepticum				*Mycoplasma synoviae*			
	Natural hair		Synthetic hair		Natural hair		Synthetic hair	
Time, h	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed
0	$\geq 1 \times 10^6$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
1	6×10^5	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	4×10^5	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
2	8×10^4	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	2×10^4	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
4	6×10^5	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	6×10^1	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
8	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
24	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
48	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
72	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$
96	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$\geq 1 \times 10^6$	$<1 \times 10^1$

organisms dropped below a count of 1×10^1/mL of culture soon after contact with the disinfectant shampoo (0 h) as measured by the CCU method (Table 1). Synthetic hair extensions in situ are difficult to wash because of the way in which they are stitched onto the natural hair, but the results presented here prove that proper washing effectively kills the microorganisms on both hair types.

Mycoplasma gallisepticum Survival on Unwashed Natural Hair and Synthetic Hair

Unwashed natural hair seeded with a titer of approximately 1×10^6/mL of viable Mg cells decreased to 6×10^5/mL by 4 h postseeding, but no viable Mg cells were detected on natural hair from 8 h onward. In contrast, the titer on synthetic hair did not decline from the initial 1×10^6/mL seed dose up to 96 h postseeding (Table 1), and, in fact, viable Mg and Ms cells were still detectable at 9 d postinfection (data not shown).

Mycoplasma synoviae Survival on Unwashed Natural Hair and Synthetic Hair

The seed dose of 1×10^6/mL cells declined logarithmically to 4×10^5/mL at 1 h, 2×10^4/mL at 2 h, and 6×10^1/mL at 4 h postseeding. No growth was detected by the CCU method for Ms on unwashed natural hair beyond 4 h postseeding. In contrast, viable Ms titers did not decline on the synthetic hair sample, even after 96 h.

qPCR

A published method was adapted to a single-tube duplex detection method for the simultaneous detection of Mg and Ms. Only unwashed hair samples were tested by qPCR, as demonstrated by the CCU method that the disinfectant shampoo-treated samples had killed all mycoplasmas soon after contact.

Proliferation was not anticipated, as seeded hair samples were dried and incubated at room temperature (i.e., no growth requirements were provided), but Mg- and Ms-specific qPCR was conducted to confirm that the color-change was due to the presence of Mg or Ms and not contaminating bacteria and also to confirm that proliferation could not explain the results obtained for the synthetic hair with the CCU method. The Mg standard curve slope was −3.42 with a y-intercept of 42.162, R^2 of 0.999, and amplification efficacy of 95.9%. The R^2 value indicates the closeness of fit between the standard curve regression line and the indicated cycle threshold (**Ct**) data points from the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. The Mg genomic DNA (**gDNA**) detection on hair did not vary greatly, and ranged from 2.12×10^5/mL to 6.53×10^4/mL for natural hair with Ct values in the order of 23.8 to 25.6, and 1.95×10^5/mL to 8.4×10^4/mL for synthetic hair with Ct values in the order of 24.05 to 26.31 (Table 2).

For Ms, a similar trend was observed with gDNA quantities 2.59×10^8/mL to 3.90×10^6/mL for natural hair with Ct values ranging from 23.91 to 30.7 and gDNA quantities of 6.4×10^7/mL to 5.85×10^6/mL for synthetic hair with Ct values ranging from 26.21 to 31.48. The standard curve used for Ms quantitation produced a slope of −3.75, a y-intercept of 54.831, R^2 of 0.999, and amplification efficacy of 87%.

The qPCR results of gDNA detection confirmed that no proliferation of Mg or Ms had occurred on the synthetic hair samples. Genomic DNA were detectable at a roughly similar quantities over the course of several days, even though viable organisms were no longer present, demonstrating that the prolonged survival of Mg and Ms on synthetic hair was purely due to maintenance of viability and not proliferation. This is consistent with experiments in diagnostic laboratories where mycoplasma DNA is detected although no viable organisms remain for culture. The 1 to 2 log reductions possibly represent disintegration of the cells following death.

Hairdressing products contain quaternary ammonium compounds known to have antimicrobial proper-

Table 2. Results of quantitative real-time duplex PCR on unwashed hair samples (per milliliter of cells pelleted from culture)

	Mycoplasma gallisepticum				*Mycoplasma synoviae*			
	Natural hair		Synthetic hair		Natural hair		Synthetic hair	
Time, h	Ct[1]	Quantity	Ct	Quantity	Ct	Quantity	Ct	Quantity
0	ND[2]	ND	24.05	1.95×10^5	23.91	2.95×10^8	26.21	6.14×10^7
1	23.92	2.12×10^5	24.32	1.63×10^5	27.99	2.02×10^7	31.48	2.25×10^6
2	23.80	2.30×10^5	25.31	8.37×10^4	28.09	1.89×10^7	27.98	2.02×10^7
4	25.04	1.01×10^5	26.05	5.08×10^4	28.75	1.25×10^7	27.76	2.33×10^7
8	24.97	1.06×10^5	25.11	9.54×10^4	29.89	6.11×10^6	29.96	5.84×10^6
24	24.29	1.66×10^5	26.31	4.28×10^4	28.01	1.99×10^7	28.43	1.52×10^7
48	25.68	6.53×10^4	26.16	4.74×10^4	28.78	1.22×10^7	29.94	5.94×10^6
72	24.61	1.37×10^5	25.87	5.81×10^4	30.38	4.49×10^6	27.98	2.03×10^7
96	24.97	1.05×10^5	25.30	8.40×10^4	30.70	3.90×10^6	28.89	1.14×10^7

[1]Ct = cycle threshold value.

[2]ND = not done, sample leak.

ties (Christensen et al., 1994) and colorants contain, among other chemicals, hydrogen peroxide and ammonia. Resolute efforts were made to ensure that all traces of colorant and shampoo were rinsed from the natural hair samples, but we cannot be certain that trace amounts of chemicals were not present. When natural hair was incubated in MB and then removed, and this MB was used to culture Mg and Ms, inhibition of growth was observed (data not shown). Alternatively, it is also possible that human hair is innately toxic to Mg and Ms. It has already been demonstrated that the normal hair scalp hair follicle epithelium possesses a functional antimicrobial defense system that includes antimicrobial peptides RNase 7, psoriasin, and Toll-like receptors 4 and 5 (Reithmayer et al., 2009). Keratin-derived antimicrobial peptides secreted by the epithelial cells of human cornea were also demonstrated to have bactericidal activity (Tam et al., 2012). Scope exists for further studies on the possibility that human hair produces antimicrobial peptides that are active against Mg and Ms.

The extended survival of both Mg and Ms on synthetic kanekalon hair fibers was an unexpected result that raises the question of whether attachment to a surface is a prerequisite for the survival and persistence of Mg and Ms in the extra-host environment. The adherence of Mg to glass and a plastic Petri dish was first reported almost half a century ago (Taylor-Robinson and Manchee, 1967). Similarly, Mg seemed to grow to a higher density in the presence of biosilon microcarrier beads, manufactured for the purpose of cultivating anchorage-dependent tissue culture cells, but did not generally affect the recovery or growth rate. Initiation of Mg culture could be accomplished by the transfer of one bead from a microcarrier culture (Freidlin, 1983). Future studies should be aimed at determining the maximum amount of time that Mg and Ms are able to survive on kanekalon fiber, differences in survival with other field strains of Mg and Ms and with other fiber types used in the manufacture of hair extensions (toyokalon, monofiber), or whether other types of plastics commonly found in the poultry house offer similar survival advantages to mycoplasmas.

ACKNOWLEDGMENTS

This study was funded by the Southern African Poultry Association (Johannesburg).

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