Polymerase chain reaction-based discrimination of viable from non-viable *Mycoplasma gallisepticum*

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The present study was based on the reverse transcription polymerase chain reaction (RT-PCR) of the 16S ribosomal nucleic acid (rRNA) of *Mycoplasma* for detection of viable *Mycoplasma gallisepticum*. To determine the stability of *M. gallisepticum* 16S rRNA *in vitro*, three inactivation methods were used and the suspensions were stored at different temperatures. The 16S rRNA of *M. gallisepticum* was detected up to approximately 20–25 h at 37 °C, 22–25 h at 16 °C, and 23–27 h at 4 °C. The test, therefore, could detect viable or recently dead *M. gallisepticum* (< 20 h). The RT-PCR method was applied during an *in vivo* study of drug efficacy under experimental conditions, where commercial broiler-breeder eggs were inoculated with *M. gallisepticum* into the yolk. Hatched chicks that had been inoculated *in ovo* were treated with Macrolide 1. The method was then applied in a flock of day 0 chicks with naturally acquired vertical transmission of *M. gallisepticum*, treated with Macrolide 2. Swabs of the respiratory tract were obtained for PCR and RT-PCR evaluations to determine the viability of *M. gallisepticum*. This study proved that the combination of both PCR and RT-PCR enables detection and differentiation of viable from non-viable *M. gallisepticum*.

Introduction

Mycoplasmas are the smallest self-replicating prokaryotes and belong to the class Mollicutes, Order I, Mycoplasmatales, family Mycoplasmataceae (Razin 1992; Razin, Yogev & Naot 1998). Mycoplasmas are devoid of cell walls and bounded by only a plasma membrane (Baseman & Tully 1997; Razin 1992); they are therefore resistant to antibiotics that affect cell wall synthesis (Kleven 2003). Some mycoplasmas are host specific, whilst others may be able to infect several species of animals. Mycoplasmas colonise only mucosal surfaces, where most species remain noninvasive, but others such as *Mycoplasma gallisepticum* are able to penetrate cells (Kleven 2003). Avian mycoplasmosis has been reported to include chronic respiratory disease, infectious sinusitis and infectious synovitis, which result in decreased egg production, reduced growth rate and decreased hatchability in poultry (Ley *et al.* 1997). The species of economic importance in poultry that are pathogenic are *M. gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis* and *Mycoplasma iowae*. *Mycoplasma gallisepticum* infection causes chronic respiratory disease in chickens. The disease is often complicated by other microorganisms, including respiratory viruses and *Escherichia coli*, which lead to severe air sacculitis and complicated chronic respiratory disease (Ley 2003).

The transmission of *M. gallisepticum* can be both vertical and horizontal. Vertical transmission of *M. gallisepticum* has been known to occur in eggs laid by infected hens. Horizontal transmission of *M. gallisepticum* occurs through direct contact between infected and susceptible chickens, especially in flocks with high population density (OIE 2004). Indirect transmission can occur through contaminated aerosol droplets, dust, feathers, fomites and farm personnel. Since *M. gallisepticum* can be transmitted vertically, maintaining a *M. gallisepticum*-free flock is only possible by obtaining *M. gallisepticum*-free chicks or eggs from an *M. gallisepticum*-free breeder flock. Practising good farm biosecurity and establishing a flock health programme, such as vaccination and prophylactic antibiotics, may also prevent *M. gallisepticum* infection (Kleven 1997). Although these are practised on many farms, *M. gallisepticum* still occurs in Malaysia where this study was carried out.

The conventional way of detecting bacterial viability depends on the ability of the bacteria to grow actively and form visible colonies on solid media. The number of viable bacteria may be severely underestimated by this method, as sub-lethally damaged bacteria (Blackburn & McCarthy 2000), fastidious and/or uncultivable bacteria (Ward, Weller & Bateson 1990) and viable cells that may lose their ability to form colonies under culture conditions will not be detected. Alternative methods for determining viability are metabolic activity and nucleic acid-based analyses (Del Mar Lleo *et al.* 2000; McCarty & Atlas 1993; Sheridan *et al.* 1998). A

wide range of molecular targets have been utilised in determining bacterial species present in samples, especially polymerase chain reaction (PCR). However, these assays do not give any indication of the viability of the bacteria. Deoxyribosomal nucleic acid (DNA), which has a very long half-life compared to ribosomal nucleic acid (RNA) (Belasco & Higgins 1988), is a rather stable nucleic acid that is detectable in live organisms and may also be detected in dead organisms due to its stability in dead cells and even in the environment (Del Mar Lleo *et al.* 2000).

The precise correlation of cell viability with detection of DNA has been shown to be poor, with DNA persisting in actively killed bacteria for significant periods of time (Masters, Shallcross & Mackey 1994). As RNA is a highly labile molecule with a very short half-life it should provide better correlation with bacterial viability compared to DNA-based assays. Under a bacteria-killing regimen, RNA has been found to positively correlate with viability (McKillip, Jaykus & Druke 1998). As DNA of dead cells may also be amplified, the detection of *M. gallisepticum* by PCR is not a direct indication of its viability or infectivity (Josephson, Gerber & Pepper 1993); it shows that the organism was present, due to either a current or a former infection that had occurred at an uncertain time. Because only viable mycoplasmas are of concern as a potential source of infection, their detection is of the utmost importance. Since ribosomal RNA (rRNA) is less stable than DNA (McKillip *et al.* 1988) and is constitutively expressed, it is considered to be a suitable target for development of a reverse transcription-polymerase chain reaction (RT-PCR) for detection of viable *Mycoplasma* cells (Marois *et al.* 2002). This would assist in determining mycoplasmacidal drug efficiency and also the *in vivo* minimum inhibitory concentration.

Mycoplasma infection is thought to be a lifelong infection by many, since a previously infected flock that has clinically recovered from the infection can have a recurrence of mycoplasmosis when the flock is challenged by other complicating viral infections (Kempf *et al.* 1994). Currently, there is still lack of information on the viability of *M. gallisepticum* after antibiotic treatment, therefore the true efficacy of drugs in eliminating *M. gallisepticum* infections remains unknown. Therefore, the objectives of this study were: to determine the stability of 16S rRNA after *M. gallisepticum* death *in vitro* under several temperature conditions; to detect viable and non-viable *M. gallisepticum* using PCR and RT-PCR in combination with an *in vivo* study of drug efficacy; and to apply this method of detection under field conditions.

Materials and methods

Mycoplasma strains

Mycoplasma gallisepticum strains used in this study were as follows: *M. gallisepticum*-S6, a reference strain obtained from the Veterinary Research Institute (VRI), Ipoh, Perak, Malaysia; ts-11, a vaccine strain; H21-11T, a local field strain isolated from an infected normal chick; and I 29, a local field strain isolated from an apparently healthy chicken.

In vitro verification of stability of 16S rRNA after mycoplasma death

The stability of 16S rRNA was determined using an *M. gallisepticum* reference strain (*M. gallisepticum*-S6), a vaccine strain (ts-11) and a field strain (H21-11T). Three methods were used to kill the mycoplasmas. Three suspensions of *M. gallisepticum* were prepared as follows: 1 mL of *M. gallisepticum* culture of each strain (*M. gallisepticum*-S6, ts-11 and H21-11T) was centrifuged at 12 000 x g for 20 min using an Eppendorf centrifuge (Eppendorf 5403, Hamburg, Germany) and the supernatant was discarded. In the first method, glycerol was added to the pellet and thoroughly re-suspended (to prevent cell lysis) and incubated at 95 °C for 15 min, as described by Marois *et al.* (2002). The second method involved re-suspension of the pellet in double-distilled water (to cause osmotic shock) and incubated for 1 h at 60 °C, as described by Marois *et al*. (2002). The third method consisted of re-suspending the resultant pellet in phosphate buffer saline and subjecting it to ultrasonic lysis for 15 min.

To confirm that the mycoplasmas were already dead, the suspensions were inoculated into a 'pleuropneumonia-like organism' (PPLO) broth (mycoplasma broth, as described by Tan [2004]) immediately after cell lysis and incubated at 37 °C. The suspensions of each strain and each method were stored at 4 °C, 16 °C and 37 °C. Samples were collected from each suspension for DNA and RNA extraction immediately before cell lysis and at 0 h, 1 h, 5 h, 15 h, 20 h, 22 h, 23 h, 24 h, 25 h, 27 h and 48 h after cell lysis. The DNA samples were evaluated using PCR. The times (h) when rRNA was last detected by PCR for each method and for storage at different temperatures were recorded for the three *M. gallisepticum* strains, and the means were calculated.

Molecular detection of viable and non-viable *Mycoplasma gallisepticum*

The PCR procedures were performed according to the method described by Marois *et al*. (2002) with some modifications. Polymerase chain reaction evaluation was performed to confirm the presence of *M. gallisepticum* before an RT-PCR was carried out to evaluate the viability of *M. gallisepticum*. Genomic DNA and RNA (total nucleic acid) were extracted using a commercially available kit, MasterPure™ Complete DNA & RNA Purification Kit from Epicentre® Biotechnologies (Wisconsin, USA), according to the method recommended by the manufacturer. Polymerase chain reaction amplification was performed with 25 µL of reaction mixture containing 5x PCR buffer, 25 mmol *M. gallisepticum* Cl_2, 100 mmol deoxynucleoside triphosphates, 0.1 µm of each primer, 2.5 units Taq DNA polymerase (Vivantis, Malaysia), PCR-grade water and 2 µL of extracted total nucleic acid. The PCR reaction procedure consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and ended with one cycle of final extension at 72 °C for 10 min.

After confirming the presence of *M. gallisepticum* infection, the viability of *M. gallisepticum* was evaluated by RT-PCR. Polymerase chain reaction was also performed on the RNA templates to rule out the possibility of any DNA contamination that would give a false positive result, because the same set of primers was used in both PCR and RT-PCR. DNase (Promega, Madison, Wisconsin, USA) treatment of RNA samples prior to RT-PCR was performed, as recommended by the manufacturer. Repeat DNA removal was needed if the PCR on the DNase treatment of RNA samples was found to be positive. The RNA templates were amplified in an automatic thermal cycler (MyCycler, BioRad, California, USA). The reaction volume was set up in a 25 µL reaction master mixture using reagents in an All-in-one RT-PCR Kit (Mbiotech, Inc., Seoul, Korea) with 2 µL of template. The RT-PCR reaction procedure consisted of reverse transcription at 48 °C for 40 min, reverse transcriptase inactivation and pre-denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and ended with one cycle of final extension at 72 °C for 10 min. Polymerase chain reaction amplicons obtained from DNA and cDNA templates were separated by using 1.5% agarose gel electrophoresis to detect genomic DNA. The sizes of the amplified PCR product were compared using a 100 bp DNA ladder.

In vivo experimental study of drug efficacy

Commercial broiler-breeder eggs were obtained from a farm in Malaysia with a history of killed vaccine vaccination in the breeder flock. The eggs from the farm were divided into two groups: an *M. gallisepticum*-inoculated and an uninoculated group (control group). Intra-yolk inoculation with *M. gallisepticum* strain H 21-11T was performed at day 6 of incubation with an *M. gallisepticum* concentration of 10^3 CFU/mL. The eggs were incubated and allowed to hatch.

All hatched chicks were wing tagged. The chicks were reared in separate cages in an experimental house as follows: amongst all the inoculated chicks that hatched, chicks that survived subsequently received antimicrobial treatment. The chicks were fed with mash concentrate with low bacterial counts and no added antibiotics (purchased from VRI, Ipoh, Malaysia). The chicks were observed daily for clinical signs of mycoplasmosis.

The first sampling was carried out on day-old chicks. Two chicks were randomly chosen from each group. Choanal cleft swabs were obtained from the chosen chicks and two swabs from the same group were pooled as one sample. Swab samples were immediately stored in PPLO broth that had been mixed with an equal volume of sterile glycerol and kept at -80 °C. The samples from the first sampling day were processed by extraction and amplification of DNA and RNA using PCR and RT-PCR, respectively, to verify the presence of *M. gallisepticum* before treatment was given. Subsequent samplings were performed on days 6, 7, 8, 9, 10 and 11 post hatching, and treatment was given on days 6, 7 and 8 post hatching. Two chicks were randomly chosen from each group for each sampling that included pooled choanal cleft swabs from each group, as described previously. The storage and processing of samples collected were as described above.

The antimicrobial used was a macrolide (labelled as Macrolide 1 in this study). Treatment was given for three consecutive days in the drinking water at the dose recommended by the manufacturer. Five out of nine *in ovo* inoculated chicks were given drug treatment, whilst the remaining four were left untreated. The treated and untreated chicks were kept in separate cages in the experimental house. Four chicks from the *in ovo* un-inoculated group served as the control group for this experiment.

In vivo study of drug efficacy under field conditions

Sacrificed chicks were obtained from a commercial broiler farm in Malaysia. The day-old chicks showed gasping and were sacrificed to find the reason behind this. The gasping chicks were shown to have caseous air sac lesions. The sacrificed chicks, which were kept on ice, were sent to the laboratory on the same day. *Post mortem* examinations were carried out immediately after arrival of the chicks. Ten chicks were sacrificed on the farm on each sampling day. The first sampling was carried out on day-old chicks. Choanal cleft, tracheal and air sac swabs were obtained from each chick and the three swabs collected from each chick were pooled as one sample. Swab samples collected were immediately stored in PPLO broth that had been mixed with an equal volume of sterile glycerol and kept at -80 °C. The samples from the first day sampling were processed by extraction and amplification of DNA and RNA using PCR and RT-PCR, respectively, to verify the presence of *M. gallisepticum* before treatment was given.

During the period of verification, culling of poor-quality chicks was carried out on the farm. Treatment was given on days 3, 4 and 5 post hatching. Subsequent sampling of 10 sacrificed chicks was performed on day 8 post hatching. No sampling was performed during the treatment period. The same sampling methods were applied. A macrolide was used as the antimicrobial in this study (labelled Macrolide 2). Three days of treatment were given via the drinking water at the dose recommended by the manufacturer.

Interpretation of results

The amplicons derived from the primers used in the present study were designed to have a molecular size of 186 bp, that is, in proximity to the 200 bp marker following agarose gel electrophoresis.

A positive PCR result indicated presence of *M. gallisepticum* DNA, but not whether *M. gallisepticum* was viable or not. A negative PCR result suggested possible absence of *M. gallisepticum* DNA in the sample, although this depended on test sensitivity. A positive RT-PCR result indicated presence of viable *M. gallisepticum*, or recently inactivated *M. gallisepticum* (less than 20 h previously, depending on

sampling, transport and storage conditions). A positive PCR, negative RT-PCR result indicated the presence of *M. gallisepticum* DNA, but absence of RNA associated with the presence of inactivated organisms (inactivation having occurred more than 20 h previously, depending on sampling, transport and storage conditions). Negative PCR and RT-PCR indicated likely absence of *M. gallisepticum* DNA/RNA, and thus, the likely absence of the organism.

Ethical considerations

This work was conducted with the approval and financial support of the Universiti Putra Malaysia and Ministry of Science, Technology and Innovation (MOSTI), project number 02-01-04-SF0370.

Results

In vitro verification of stability of 16S rRNA after mycoplasma death

The stability of 16S rRNA was evaluated by RT-PCR for the times shown in Table 1. The time when rRNA was last detected ranged from 20.3–25.0 h when stored at 37 °C, 22.7–25.7 h when stored at 16 °C; and 23.0–27.0 h when stored at 4 °C. Different methods of inactivation preserved rRNA for different durations. Of all the storage temperatures (4 °C, 16 °C and 37 °C), ultrasonic inactivation allowed preservation of rRNA for the longest time, followed by osmotic and heat inactivation and, lastly, heat inactivation alone. A decrease in time of rRNA preservation was observed when the storage temperature increased. By storing under 4 °C compared to 37 °C, the rRNA preservation time was increased by 2 h when ultrasonic inactivation was performed, 2 h when osmotic and heat inactivation were performed and 3 h when heat inactivation alone was used. All PCR results on rRNA were negative, indicating absence of DNA contamination following DNase treatment.

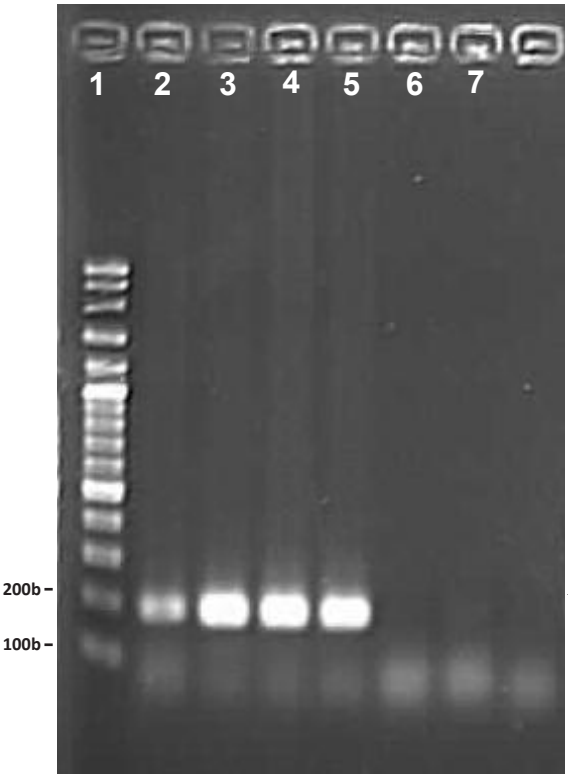
In vivo study of drug efficacy under experimental conditions

The PCR evaluation to verify the presence of *M. gallisepticum* DNA on day 0 indicated that *M. gallisepticum* infection was absent in the control group but present in both treated and untreated groups (bands from wells 2 to 6), as shown in Figure 1. The same results were obtained from day 6 to day 8 (treatment given) and day 9 to day 11 post hatch (post treatment period), indicating the presence of the organism in the chicks up to day 11 post hatch.

The RT-PCR evaluation of viability of *M. gallisepticum* after Macrolide 1 treatment revealed that *M. gallisepticum* remains viable from the first day of treatment up to day 2 post treatment (day 10 post hatch), as shown in Figure 2, where bands were present in both treated and untreated groups (wells 3 to 6). However, on day 3 post treatment (day 11 post hatch), one of the treated group showed a negative result (no band; well 8) for RT-PCR evaluation.

In vivo study of drug efficacy under field conditions

The PCR evaluation to verify the presence of *M. gallisepticum* infection at day 0 indicated that all 10 day 0 chicks that were sacrificed were *M. gallisepticum* infected, as shown in Figure 3, where bands were present from wells 3 to 12, indicating that the infection was indeed vertically transmitted from hen to eggs. The RT-PCR evaluation to verify the viability of *M. gallisepticum* at day 0 indicated that the *M. gallisepticum* detected in the chicks was all viable *M. gallisepticum*, as shown in Figure 4, where bands were present from wells 3–12.



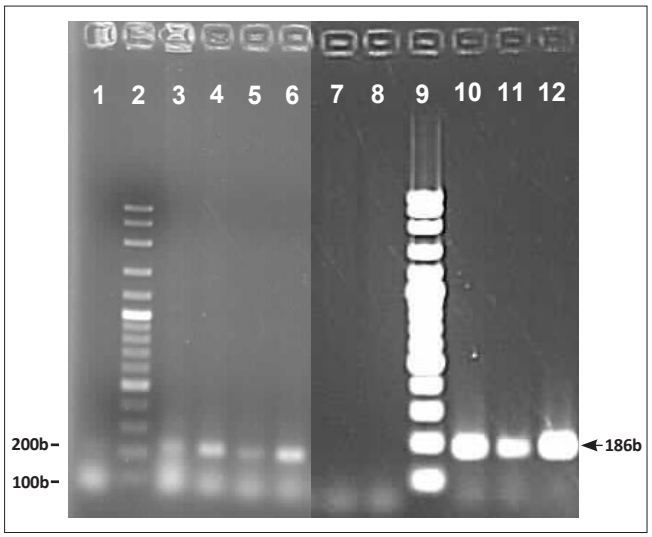
Source: Authors' own creation
Well 1, marker; wells 2 and 3, treatment groups; wells 4 and 5, non-treatment groups; well 6, control group; well 7, PCR control.

FIGURE 1: Agarose gel electrophoresis of polymerase chain reaction amplified experimental samples obtained on day 0.

TABLE 1: The mean hour when rRNA was last evaluated by reverse transcription polymerase chain reaction when inactivated using three different methods and stored at 37 °C, 16 °C and 4 °C.

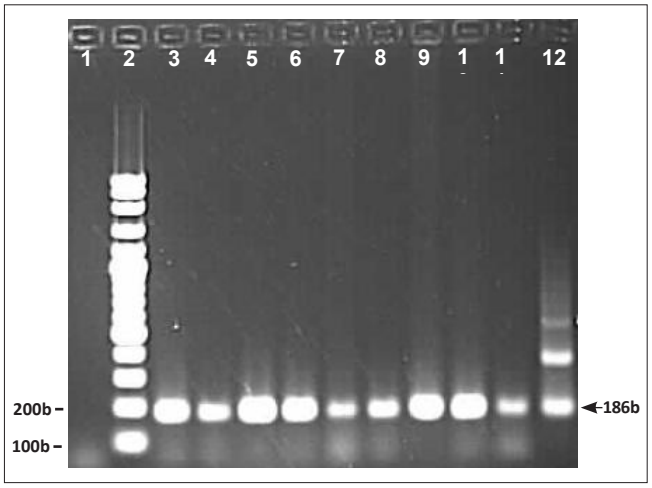
Strains	Osmotic lysis and Heat lysis			Ultrasonic lysis			Heat lysis		
	37 °C	16 °C	4 °C	37 °C	16 °C	4 °C	37 °C	16 °C	4 °C
M. gallisepticum-S6	23	23	25	25	27	27	23	23	23
M. gallisepticum ts-11	25	27	27	23	25	27	15	22	23
M. gallisepticum H21-11T	23	23	25	27	27	27	13	23	22
Mean	23.7	25.0	25.7	25.0	25.7	27.0	20.3	22.7	23.0

M. gallisepticum, *Mycoplasma gallisepticum; M. gallisepticum*-S6, Reference strain; *M. gallisepticum* ts-11, Vaccine strain; *M. gallisepticum* H21-11T, Field strain.



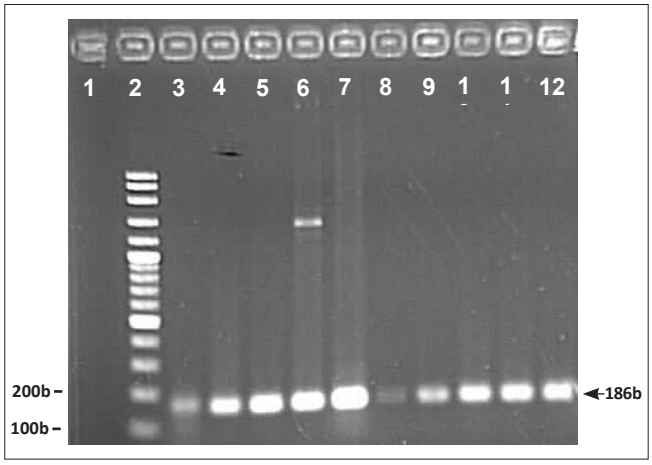
Source: Authors' own creation
Wells 1 and 7, control group; wells 3 and 4 (day 2 post Macrolide 1 treatment), 8 and 10 (day 3 post Macrolide 1 treatment), treated groups; wells 5 and 6, 11 and 12, untreated groups.

FIGURE 2: Agarose gel electrophoresis of reverse transcription polymerase chain reaction amplified experimental samples obtained on day 10 post hatch (wells 1–6) and day 11 post hatch (wells 7–12).



Source: Authors' own creation
Well 1, PCR control; well 2, marker; wells 3 to 12, samples.

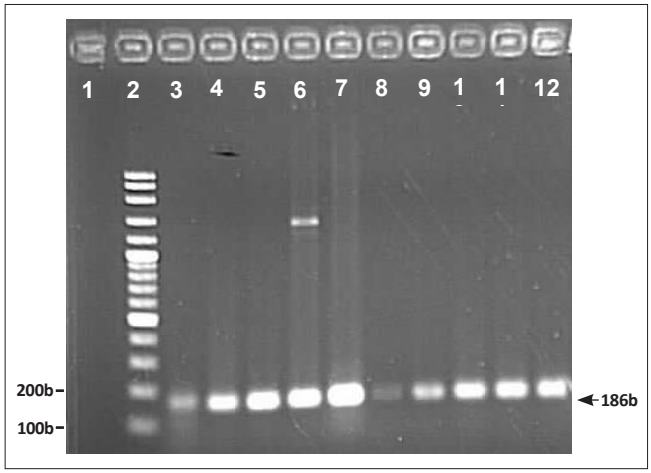
FIGURE 3: Agarose gel electrophoresis of polymerase chain reaction amplified field samples obtained on day 0.



Source: Authors' own creation
Well 1, PCR control; well 2, marker; wells 3 to 12, samples.

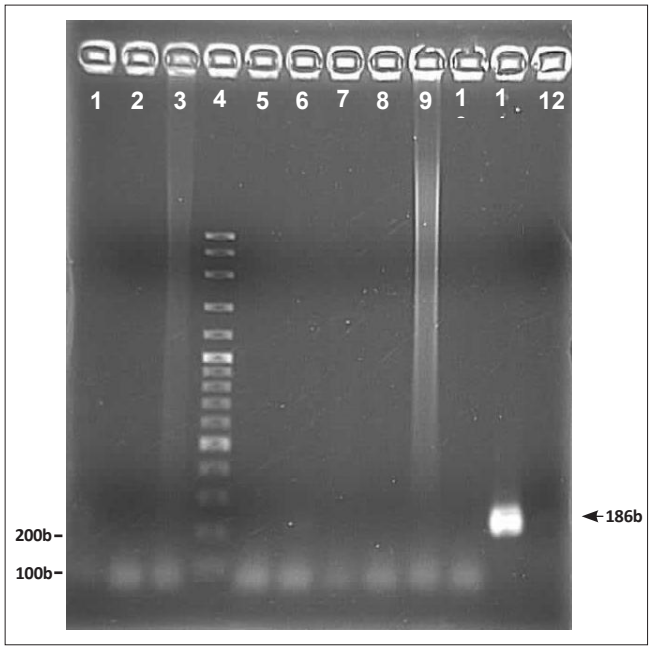
FIGURE 4: Agarose gel electrophoresis of reverse transcription polymerase chain reaction amplified field samples obtained on day 0.

The PCR evaluation of the samples obtained at the second sampling on day 3 post treatment with Macrolide 2 (day 8 post hatch) did not initially have any bands on agarose gel electrophoresis. It was suspected that the negative result was indeed a false negative result due to low *M. gallisepticum* load; therefore, another PCR amplification was performed using a larger volume of DNA template and amplified for 40 cycles instead of the initial 30 cycles. The result of the second attempt is shown in Figure 5, when all 10 chicks sampled were *M. gallisepticum* infected (bands from wells 3 to 12). The RT-PCR evaluation of samples obtained on day 3 post treatment with Macrolide 2 had a negative result, indicating that the *M. gallisepticum* was not viable in the chicks sampled, except for one where *M. gallisepticum* was still viable (band at well 11), as shown in Figure 6.



Source: Authors' own creation
Well 1, marker; well 2, PCR control; wells 3 to 12, chicks treated with Macrolide 2.

FIGURE 5: Agarose gel electrophoresis of polymerase chain reaction amplified field samples obtained on day 3 post treatment with Macrolide 2.



Source: Authors' own creation
Well 4, marker; well 1, PCR control; wells 2, 3, 5 to 12, chicks treated with Macrolide 2.

FIGURE 6: Agarose gel electrophoresis of reverse transcription polymerase chain reaction amplified field samples obtained on day 3 post treatment with Macrolide 2.

Discussion

During the RNA extraction for RT-PCR amplification, two successive DNA removals were performed in the present study. This has a high tendency to cause further degradation of RNA due to the processing temperature, and the protein removal protocols may further dilute the concentration of the RNA template, so that a less than satisfactory amount of product is produced by RT-PCR amplification. A better way to remove DNA contamination is suggested whereby the volume of DNase enzymes is doubled and the incubation time in the water bath is increased instead of performing the protocol twice.

The 16S rRNA of *M. gallisepticum* was most stable when *M. gallisepticum* was inactivated using ultrasonic lysis, less stable using osmotic and heat lysis (60 °C, 1 h), and least stable using heat lysis alone (95 °C, 15 min). This finding coincides with the findings of Marois *et al.* (2002), where rRNA was last detected at 23 h when the samples were inactivated by osmotic shock and heat treatment, and 20 h when the samples were inactivated by heat inactivation alone. Both were stored at room temperature after inactivation. The differences in 16S rRNA stability observed amongst the three inactivation methods could be explained by the degree of RNase enzymes denaturation in each inactivation technique (Marois *et al.* 2002). A higher incidence of RNase denaturation leaves fewer enzymes to digest RNA; therefore, RNA persists longer in dead cells in such samples. From the results it can be said that the most effective inactivation of RNase occurred with ultrasonic lysis, which generates the highest intensity of heat, rather than during heat inactivation for 1 h at 60 °C, and least effective when inactivated at 95 °C for 15 min. Based on this, it is suggested that the persistence of rRNA in dead cells might depend on various conditions present at the time of mycoplasma death, which could influence the degree of RNA exposure to RNases and the rate of RNA degradation. The results obtained in the present study confirm the results obtained by Marois *et al*. (2002) on detection of 16S rRNA stability of mycoplasma cells. The results obtained in this study also confirm the results obtained by Sheridan *et al*. (1998) and Kempsell and Kwok (1990) on detection of bacterial rRNA using an RT-PCR, which suggest the presence of live or recently dead bacteria.

In field conditions, the temperature in the choanal cleft of chickens is approximately 30 °C. RNases are unlikely to be inactivated when compared to post treatment with ultrasonic lysis at 60 °C or 95 °C. For this reason (and most probably coupled with other reasons such as presence of RNases from other less fragile bacteria), the stability of free rRNA of mycoplasma in field conditions is suggested to be less than 20.3 h (the shortest time for degradation of rRNA in the present study). The increase in the duration of rRNA detection by 2–3 h when the storage temperature was decreased from 37 °C to 4 °C with all the inactivation methods suggests that persistence of rRNA in dead cells also depends on the storage temperature. It is therefore recommended that samples collected for the evaluation of *M. gallisepticum* viability should be stored on ice and sent to the laboratory within 20 h after sample collection. The time of sample collection should be clearly stated to ensure that samples are processed within the time limit of 20 h after sample collection.

The artificial induction of vertical transmission of *M. gallisepticum* using intra-yolk inoculation resulted in very low hatchability in commercial broiler-breeder embryonated eggs. It was postulated that the extremely low hatchability was due to the concentration of *M. gallisepticum* that was inoculated *in ovo*. It is therefore recommended that a hatchability test should be carried out to determine a suitable *M. gallisepticum* concentration if artificial induction of vertical transmission were to be performed. On day 3 post treatment, one of the treated pairs showed a negative result on RT-PCR evaluation, meaning that these chicks had non-viable *M. gallisepticum* that had been inactivated by Macrolide 1 at least 20 h before sampling. However, another group of chicks treated with Macrolide 1 still possessed viable *M. gallisepticum*, so the efficacy of Macrolide 1 could not be confirmed at day 3 post treatment.

Upon RT-PCR evaluation in the field study, only one chick was shown to have viable *M. gallisepticum*. The remaining chicks had inactivated or dead *M. gallisepticum*. This means that Macrolide 2 had succeeded in inactivating *M. gallisepticum* in most of the chicks that were sampled. It is recommended that future studies on *in vivo* drug efficacy should have a longer duration and larger sample size in order to thoroughly evaluate the efficacy of antimicrobial drugs against *M. gallisepticum* infection. It is also recommended that a more sensitive technique, such as Real Time PCR, should be used to prevent incidence of false negatives, as occurred in the present study.

Conclusion

In conclusion, the present study shows that the stability of *M. gallisepticum* 16S rRNA can be evaluated up to 20–25 h at 37 °C, 22–25 h at 16 °C and 23–27 h at 4 °C. Therefore, RT-PCR enables the detection of viable or recently dead (less than 20 h) *M. gallisepticum*. RT-PCR evaluation of 16S rRNA of *M. gallisepticum* enables detection of viable *M. gallisepticum*. PCR enables detection of both viable and non-viable *M. gallisepticum*, without differentiating between them. The combination of both PCR and RT-PCR enables detection and differentiation to some degree of viable and non-viable *M. gallisepticum*. Thus, both methods should be used for detection and determination of viable *M. gallisepticum* in suspected cases. RT-PCR of 16S rRNA of *M. gallisepticum* is also found to be suitable and applicable under field conditions.

Acknowledgements

Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

A.I. (Universiti Putra Malaysia) was the project leader, C.G.T. (Universiti Putra Malaysia) and S.H.K (University of Georgia) were responsible for experimental and project design. C.G.T. and C.P.Y. (Universiti Putra Malaysia) performed all of the experiments. A.R.O. (Universiti Putra Malaysia) made conceptual contributions.

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