RESEARCH REPORT
SWINE HEALTH

Title: Optimization and viability of lung homogenate for successful *Mycoplasma hyopneumoniae* exposure in gilts during acclimation – NPB #19-120

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Industry summary: Gilt acclimation has become a common practice in the United States swine industry to control *Mycoplasma hyopneumoniae* infections. The goal of gilt acclimation is to achieve uniform exposure of gilts prior to entry to the breeding herd, to create a "Day 0" for disease elimination purposes. Utilization of *M. hyopneumoniae*-positive lung homogenate (LH) for gilt exposure has shown to be efficient. However, the characterization, preparation, handling, and storage of the LH has not been tested experimentally, which poses a risk for failure of successful exposure. Therefore, the purpose of this study was to establish methods of LH preparation and storage to optimize the process and result in a successful exposure of gilts during acclimation. Three aims were completed in this study, including: 1) To evaluate the *M. hyopneumoniae* concentration in different anatomical lung sections of naturally infected pigs, 2) To evaluate the effect of the LH homogeneity on *M. hyopneumoniae* detection by real-time PCR, and 3) To evaluate the viability of *M. hyopneumoniae* in the LH under various storage and dilution conditions. For aim one, lung donor pigs were selected, and categorized into low, medium and high Ct value groups based on deep tracheal catheter (DTC) samples. After humane euthanasia, lung lesion scores were recorded and bronchial swabs (BS) were collected from each lung lobe. For each lobe, tissue was blended at a 70:30 proportion of tissue and Friis medium to create lung lobe-specific homogenate samples. Real-time PCR for *M. hyopneumoniae* detection was performed on BS and lung lobe homogenates. For aim two, lungs were collected from four donor pigs, and blended whole at a 70 tissue:30 Friis ratio. The resulting LH was used to obtain 50:50 and 30:70 dilutions. All three LH dilutions were further diluted at a 1:10 ratio and treated as follows: i) No filtration, ii) Gross filtration using a nylon stocking and iii) Fine filtration (100 µm). Filtered and diluted products were tested for *M. hyopneumoniae* via real-time PCR. For aim three, whole lungs were obtained from two *M. hyopneumoniae*-positive donors and blended separately. For each donor lung, two preparations were created including a 70:30 tissue:Friis medium dilution and a 70:30 tissue:saline dilution. Lung homogenate samples were then stored in different conditions: 1) Fresh at 25°C, 2) 1 week frozen at -20°C, 3) 1 month frozen at -20°C and 4) 6 months frozen at -20°C. There were four challenge groups based on the length of storage. For each challenge group, two gilts were randomly assigned a treatment of either LH prepared with saline or medium from donor pig one or two resulting in four gilts for both saline and medium. For all challenge groups, DTC and sera were collected at the start of the trial and four weeks post-inoculation. At necropsy, lung lesions were evaluated, and a BS was collected.

Gilts with low DTC Ct values had a relatively uniform distribution of *M. hyopneumoniae* throughout the whole lung, as well as a high bacterial load, compared to pigs with medium or high DTC Ct values. In addition, lung lesions were not a reliable indicator of the presence of *M. hyopneumoniae* in the lesioned lobe as *M. hyopneumoniae* was detected in lobes without any lesions and *vice versa.* Second, dilution and filtration method of the lung homogenate generated conflicting results, without a clear indication of their effect on *M. hyopneumoniae* detection by real-time PCR. Regarding the third aim of the investigation, both Friis medium and saline were adequate for dilution of LH, even when LH was frozen for up to six months at -20°C. However, the capability of LH to induce disease in gilts appeared to be slightly lower for LH that was frozen for six months, compared to LH frozen for one month or less.

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Key findings

- Lung lesion scores were not a suitable method for selecting a donor pig, as *M. hyopneumoniae* was detected in lobes without any lung lesions and vice versa.
- Selecting a pig with a low deep tracheal catheter (DTC) sample Ct value provided a relatively consistent load of *M. hyopneumoniae* throughout the whole lung, as well as a higher bacterial load, compared to pigs in the medium and high DTC Ct groups.
- Both Friis medium and saline were adequate for dilution of LH regarding viability of LH after being frozen for 6 months or less.
- The capability of the LH to induce disease appeared to be slightly lower after six months of storage in -20°C, compared to homogenate frozen for less than or equal to one month, as evidenced in the number of infected gilts and observed lung lesions.

Keywords: *Mycoplasma hyopneumoniae*, swine, gilt acclimation, lung homogenate, exposure.

Scientific Abstract

Gilt acclimation has become a common practice in the United States swine industry to control infections caused by *M. hyopneumoniae*. The goal of gilt acclimation is to achieve uniform exposure of gilts prior to entry to the breeding herd, to create a “Day 0” for disease elimination purposes. Swine practitioners utilize lung homogenate (LH) containing *M. hyopneumoniae* for exposure, which has shown to be effective in a timely manner. However, the characterization, preparation, handling, and storage of the LH has not been tested experimentally, which poses a risk for failure of successful exposure. Therefore, the purpose of this study was to establish methods of LH preparation and storage to optimize the process and result in a successful exposure of gilts during acclimation.

Three aims were completed in this study including: 1) To evaluate the *M. hyopneumoniae* concentration in different anatomical lung sections of naturally infected pigs, 2) To evaluate the effect of the LH homogeneity on *M. hyopneumoniae* detection by real-time PCR, and 3) To evaluate the viability of *M. hyopneumoniae* in the LH under various storage and dilution conditions.

For aim one, a total of nine lung donor pigs were selected from three different farms, and categorized into the following Ct value groups: low < 24 Ct, medium 25-30 Ct, and high 31-39 Ct based on deep tracheal catheter (DTC) samples. Selected pigs were humanely euthanized, followed by observation of lung lesion scores and collection of bronchial swabs (BS) from each lung lobe. For each lobe, tissue was blended at a 70:30 proportion of tissue and Friis medium to create lung lobe-specific homogenate samples. Real-time PCR for *M. hyopneumoniae* detection was performed on BS and lung lobe homogenates (5 replicates each).

For aim two, lungs were collected from four donor pigs, and blended whole at a 70 tissue: 30 Friis ratio. The resulting LH was used to obtain 50:50 and 30:70 dilutions. All three LH dilutions were further

diluted at a 1:10 ratio and treated as follows: i) No filtration, ii) Gross filtration using a nylon stocking and iii) Fine filtration (100 µm). Filtered and diluted products were tested for *M. hyopneumoniae* via real-time PCR (5 replicates each).

For aim three, whole lungs were obtained from two *M. hyopneumoniae*-positive donors and blended separately. For each donor lung, two preparations were created including a 70:30 tissue:Friis medium dilution and a 70:30 tissue:saline dilution. From those dilutions, four dilution aliquots were created and stored in different conditions: 1) Fresh at 25ºC, 2) 1 week frozen at -20ºC, 3) 1 month frozen at -20ºC and 4) 6 months frozen at -20ºC. There were four challenge groups based on the length of storage, with eight gilts each, resulting in 32 challenged gilts. For each challenge group, two gilts were randomly assigned a treatment of either LH prepared with saline or medium from donor pig one or two resulting in four gilts for both saline and medium. For all challenge groups, DTC and sera were collected at the start of the trial and four weeks post-inoculation. At necropsy, lung lesions were evaluated, and a BS was collected.

The results obtained from aim one suggested that selecting a pig with a low DTC Ct value provided a relatively consistent load of *M. hyopneumoniae* throughout the whole lung, as well as a higher bacterial load, compared to gilts in the medium and high DTC Ct groups. In addition, lung lesion scores were not a suitable method for selecting a donor gilt, as *M. hyopneumoniae* was detected in lobes without any lung lesions and *vice versa*. Results from aim two indicated that dilution and filtration of the lung homogenate generated conflicting results, without a clear indication of their effect on *M. hyopneumoniae* detection by real-time PCR. Lastly, aim three results showed that both solutions, Friis medium and saline, were adequate for dilution of LH, and the capability of the LH to induce disease appeared to be slightly lower after six months of storage, compared to homogenate frozen for less than or equal to one month. Overall, conclusions drawn from this pilot study help optimize the preparation of LH to result in successful gilt exposure for *M. hyopneumoniae* acclimation.

Introduction

Mycoplasma hyopneumoniae causes enzootic pneumonia, a chronic respiratory disease in swine known to economically impact herds worldwide.[1] To control *M. hyopneumoniae* in sow herds, gilt acclimation has become a common practice in the United States by use of *M. hyopneumoniae* exposure from seeder animals, tracheal inoculation or aerosol (known in the field as fogging). Gilt acclimation aids to achieve uniform exposure of female pigs prior to entry to the breeding herd, or to create a “Day 0” for disease elimination purposes.[2-4] One method for gilt acclimation includes the use of herd-specific lung homogenate (LH) for exposure. However, the characterization, preparation, and handling of LH has been minimally evaluated, which poses risks for exposure failure.[5] This study addressed three questions with the goal of fine-tuning LH preparation to optimize the use of LH to result in a successful exposure in gilts during acclimation. The three questions are as follows: 1) How does the concentration of *M. hyopneumoniae* differ between anatomical lung sections of naturally infected pigs? 2) How does the homogeneity of LH affect *M. hyopneumoniae* detection by real-time PCR? and 3) How is the viability of *M. hyopneumoniae* in LH altered under various storage and dilution conditions?

Objectives

This investigation consisted of a series of experiments designed to fulfill the proposed specific aims:

- Aim 1: To evaluate the *M. hyopneumoniae* concentration in different anatomical lung sections of naturally infected pigs.
- Aim 2: To evaluate the effect of the lung homogenate homogeneity on *M. hyopneumoniae* detection by real-time PCR.
- Aim 3: To evaluate the viability of *M. hyopneumoniae* in the lung homogenate under various storage and dilution conditions.

Materials & Methods

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Experimental design and methods are graphically represented in Figure 1.

Aim 1: In order to evaluate bacterial load in different anatomical lung sections, a total of nine lung donor pigs were selected from three different farms (n=3/farm). Donor pigs were selected based on Ct values from deep tracheal catheter (DTC) samples tested for *M. hyopneumoniae* by real-time PCR. Pigs were categorized into the following Ct value groups: low < 24 Ct, medium 25-30 Ct, and high 31-39 Ct. Selected pigs were humanely euthanized, followed by observation of lung lesion scores[6] and collection of bronchial swabs (BS) from each lung lobe. For each lobe, tissue was blended at a 70:30 proportion of tissue and Friis medium using a Ninja® Blender (© 2019 SharkNinja Operating LLC) to create lung lobe-specific homogenate samples. Real-time PCR for *M. hyopneumoniae* detection was performed on BS samples and lung lobe homogenates (5 replicates each). Statistical analysis was performed using ANOVA to compare Ct values of lung homogenates (LH) from different lung lobes. Additionally, the correlation between Ct values from BS, lung lobe homogenates and lung lobe lesion scores were evaluated using the Pearson coefficient.

Aim 2: In order to evaluate the effect of LH homogeneity on *M. hyopneumoniae* detection, lungs were collected from four donor pigs as previously described in aim one, and blended whole at a 70 tissue:30 Friis ratio. The resulting LH was used to obtain 50:50 and 30:70 dilutions. All three LH dilutions were further diluted at a 1:10 ratio and treated as follows: i) No filtration, ii) Gross filtration using a nylon stocking and iii) Fine filtration (100 µm). Filtered and diluted products were tested for *M. hyopneumoniae* via real-time PCR (5 replicates each). Statistical analysis was performed using ANOVA to compare Ct values of LH from different preparation conditions.

Aim 3: In order to evaluate the viability of *M. hyopneumoniae* in the LH under various storage and dilution conditions, whole lungs were obtained from two *M. hyopneumoniae*-positive donors and blended separately. The donor pigs were confirmed positive by real-time PCR from deep tracheal catheter (DTC) samples prior to euthanasia. For each donor lung, two preparations were created including a 70:30 tissue:Friis medium dilution and a 70:30 tissue:saline dilution. From those dilutions, four dilution aliquots were created and stored in different conditions: 1) Fresh at 25ºC, 2) 1 week frozen at -20ºC, 3) 1 month frozen at -20ºC and 4) 6 months frozen at -20ºC.

A 2x3 factorial study design, followed by a challenge model was used.There were four challenge groups based on the length of storage, with eight gilts each, resulting in 32 challenged gilts. For each challenge group, two gilts were randomly assigned a treatment of either LH prepared with saline or medium from donor pig one or two resulting in four gilts for both saline and medium. Before inoculation, the homogenate was further diluted with medium or saline at a 1:10 ratio and was filtered. Gilts were intra-tracheally inoculated with 10 mL of LH. For all challenge groups, DTC samples and sera were collected at the start of the trial and four weeks post-inoculation. At necropsy, lung lesions were evaluated,[6] and a BS was collected. For this study, a viable LH was defined as capable of causing *M. hyopneumoniae* infection in a single gilt. Infection was determined if a *M. hyopneumoniae* positive PCR result was obtained from a DTC or BS sample.

Results

Aim 1: Lesions were observed on all evaluated lungs. All lobe-specific bronchial swabs (BS) and 85% of lung homogenate (LH) samples were positive for *M. hyopneumoniae*, regardless of evident lung lesions. Mean Ct values in LH samples were significantly lower in low deep tracheal catheter (DTC) Ct pigs compared to medium and high DTC Ct pigs ($p < 0.0001$), along with less variation of lung homogenate Ct values across all lobes for low DTC Ct pigs (Figure 2). All LH samples were positive from pigs with low DTC Ct values, in contrast to pigs with medium and high DTC Ct values, where there were both negative and positive lung homogenates (Figure 2).

Aim 2: Dilution of the lung homogenate generated conflicting results among samples, without a clear indication of its effect on *M. hyopneumoniae* detection by real-time PCR. Regarding filtration methods, two of the four sets of samples showed a within pig significant difference in lung homogenate Ct values for the LH that were diluted at a 70:30 ratio and filtered either using a nylon stocking or fine filtration (100 µm) ($p \leq 0.046$). The third set of samples showed a significant difference in Ct values for the LH that was

diluted at a 50:50 ratio and filtered with either of the filtering methods (p≤ 0.038). The type of filtration, namely gross using a nylon stocking vs fine (100 µm), did not have a significant effect. No filtration effect was observed for the fourth set of samples.

Aim 3: All gilts were negative to *M. hyopneumoniae* prior to inoculation. Overall, 31 of the 32 gilts in the study were detected infected with *M. hyopneumoniae* after exposure. One gilt inoculated with LH stored frozen for 6 months resulted in negative DTC, BS and serum ELISA. Histopathological lesions compatible with *M. hyopneumoniae* infection were observed in all 32 gilts.[7] Refer to Table 1 for ELISA, PCR and lung lesion scoring for gilts in all experimental groups 28 days post-inoculation.

Discussion

Based on the conditions of this study, the detection of *M. hyopneumoniae* in deep tracheal catheter (DTC) samples was useful when selecting a donor pig for lung homogenate (LH) preparation. Selecting a pig with a low DTC Ct value provided a relatively consistent load of *M. hyopneumoniae* throughout the whole lung, as well as a higher bacterial load, compared to gilts in the medium and high DTC Ct groups. In addition, lung lesion scores were not a suitable method for selecting a donor gilt, as *M. hyopneumoniae* was detected in lobes without apparent lung lesions and *vice versa*.

Regarding dilution and filtration, these preliminary results point that dilution could have an effect on LH Ct value when filtration is applied, nevertheless, the sample size was insufficient to be able to draw conclusions. The lower dilutions, 70:30 and 50:50, resulted in significantly lower LH Ct values when filtration was applied compared to no filtration in three of the four sets of samples. However, the type of filtration did not appear to have a significant effect.

When evaluating the viability of *M. hyopneumoniae*, both solutions, Friis medium and saline, were adequate for dilution of LH, as both preparations infected gilts even after being stored for six months at -20ºC. However, the capability of the LH to induce disease appeared to be slightly lower after six months of storage, compared to homogenate frozen for less than or equal to one month, as evidenced in the number of infected gilts and observed lung lesions.

It is important to note that this is a pilot study and a greater sample size may be needed for definitive results. A larger sample size would yield a more accurate analysis of the observed trends.

References

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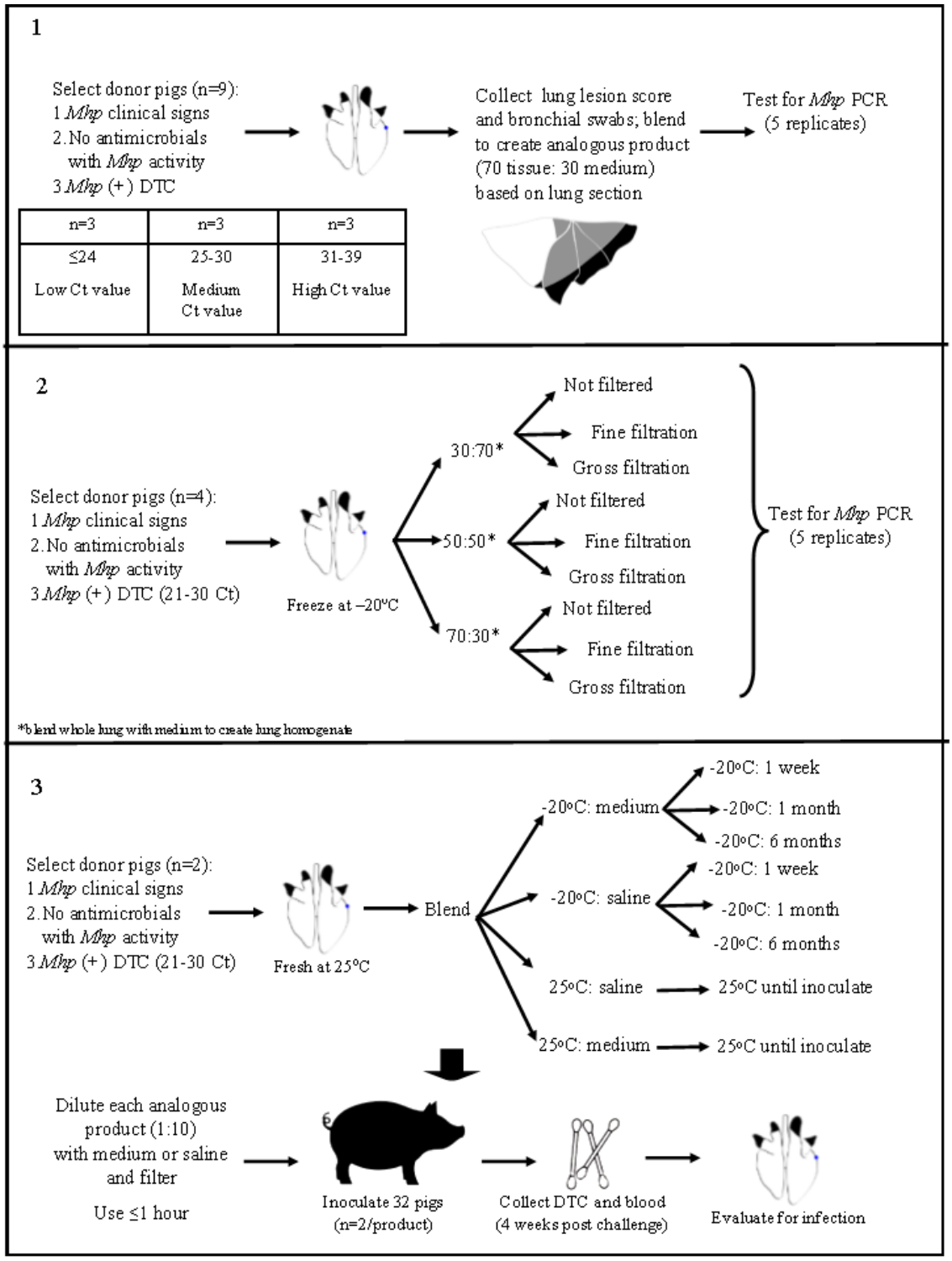

Figure 1. Study experimental design.

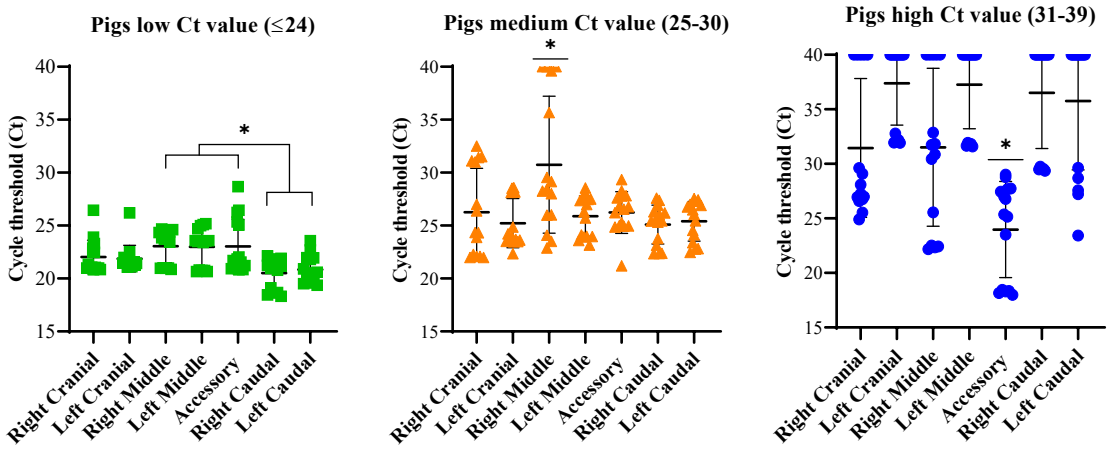

Figure 2. Cycle threshold (Ct) value distribution within each lung lobe in pigs with low (≤24), medium (25-30) and high (31-39) Ct values in deep tracheal catheter samples. Within a lung lobe, Ct values from three different pigs (5 replicates each) are represented. *Statistically significant difference in mean lung lobe Ct value ($p \leq 0.05$).

Table 1. Aim 3 ELISA, PCR and lung lesion scoring for gilts in all experimental groups 28 days post-inoculation.

		Experimental group			
Parameter	**Dilution solution**	**Fresh**	**Frozen 1 week**	**Frozen 1 month**	**Frozen 6 months**
Seropositive (%)	**Medium**	75	100	75	25
	Saline	50	100	75	0
DTC Ct (Mean ±SD)	**Medium**	18.68 (0.62)	18.43 (1.21)	19.21 (2.30)	20.86 (0.75)
	Saline	19.77 (3.98)	20.57 (2.56)	18.52 (0.73)	24.52 (9.28)
BS Ct (Mean ±SD)	**Medium**	21.03 (0.69)	21.44 (1.96)	19 (1.23)	19.09 (1.46)
	Saline	19.66 (0.70)	23.01 (2.81)	20.08 (0.41)	23.25 (10.97)
Lung Lesion (%) (Mean ±SD)	**Medium**	47 (16.57)	22 (10.23)	24.75 (14.89)	24.75 (9.95)
	Saline	28.50 (15.02)	25 (7.62)	17.75 (8.54)	13.25 (13.45)

SD=standard deviation. DTC= deep tracheal catheter. BS=Bronchial swab. Ct=Cycle threshold.