

**PERFORMANCE OF TRANSPORT AND SELECTIVE MEDIA FOR SWINE *BORDETELLA BRONCHISEPTICA*
RECOVERY AND IT COMPARISON TO POLYMERASE CHAIN REACTION DETECTION**

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ABSTRACT

Three comparative assays were performed seeking to improve the sensitivity of the diagnosis of *Bordetella bronchiseptica* infection analyzing swine nasal swabs. An initial assay compared the recovery of *B. bronchiseptica* from swabs simultaneously inoculated with *B. bronchiseptica* and some interfering bacteria, immersed into three transport formulations (Amies with charcoal, trypticase soy broth and phosphate buffer according to Soerensen supplemented with 5% of bovine fetal serum) and submitted to different temperatures (10°C and 27°C) and periods of incubation (24, 72 and 120 hours). A subsequent assay compared three selective media (MacConkey agar, modified selective medium G20G and a ceftiofur medium) for their recovery capabilities from clinical specimens. One last assay compared the polymerase chain reaction to the three selective media. In the first assay, the recovery of *B. bronchiseptica* from transport systems was better at 27°C and the three formulations had good performances at this temperature, but the collection of qualitative and quantitative analysis indicated the advantage of Amies medium for nasal swabs transportation. The second assay indicated that MacConkey agar and modified G20G had similar results and were superior to the ceftiofur medium. In the final assay, polymerase chain reaction presented superior capability of *B. bronchiseptica* detection to culture procedures.

Key words: *Bordetella bronchiseptica*, swine, transport media, selective media, PCR

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INTRODUCTION

One of the main respiratory disease entities in swine production is the rhinitis. The cause of swine rhinitis is often infectious (*Bordetella bronchiseptica*, *Mycoplasma hyorhinis*, cytomegalovirus and Aujeszky's disease virus), but ammonia and dust as well as foreign bodies can also induced mild mucosal inflammation of short duration (4). However, when toxigenic strains of *Pasteurella multocida* are present in damaged nasal mucosa, it might adhere, proliferate and subsequently provoke progressive atrophic rhinitis with permanent alteration of the nasal structure and function (4).

B. bronchiseptica is the mainly primary etiologic agent of atrophic rhinitis in swine and bronchopneumonia in young piglets and represents a major impact to swine health worldwide (26). Although clinical signs of atrophic rhinitis can be suggestive of *B. bronchiseptica* infection (4), a definitive diagnosis can be achieved by bacteriologic examination of nasal secretions (16). Swine nasal specimens are routinely shipped to laboratories under a variety of conditions: room temperature or under refrigeration, swabs immersed or not in transport medium with a transport time ranging from one to three days. Many other variables could interfere with the correct diagnosis, such as the primary isolation media employed to isolate the pathogen (13), since *B. bronchiseptica* grows more slowly than other bacteria present in the nasal cavity and the overgrowth with commensal flora during isolation procedures often occur (25).

Although bacterial culture represents a definitive diagnosis, it has some limitations. The culture processing of *B. bronchiseptica* use to consume two days and definitive tests should be lead only after subcultures (32). Even though bacteriological culture has a high diagnostic specificity, it

diagnostic sensibility is low, since only a fraction of the total bacterial species is able to grow in artificial media (7, 17). Polymerase chain reaction (PCR) is a molecular technique of nucleic acids detection sensible and specific enough to permit an accurated diagnosis of many pathogens. This technique is not limited by the capability of microorganisms of growing in cultures, besides the amplicon characterization might provide epidemiological and phylogenetic informations.

Various PCR protocols have been developed that target different regions of the *Bordetella* spp. genome: insertion sequences IS481 and IS1001 (8, 9, 28, 30), the pertussis toxin promoter region (9, 28) and the adenylate cyclase gene (5). However, greater part of *Bordetella* PCR protocol is related *B. pertussis* species. In 1999 a specific PCR for *B. bronchiseptica* detection was described, which target region of amplification encoded the structural gene of flagelin (*fla*) (14). This sequence was chosen as target since *B. bronchiseptica* is the only one, between the three main *Bordetella* species (*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*) to express the flagelin gene (32)

Currently, there are few data to support recommendations to increase the probability to detect *B. bronchiseptica* from swine nasal swabs. The purpose of this study was to establish the best conditions for the recovery of *B. bronchiseptica* from swine nasal swabs using the most efficient transport and primary isolation media. We also compared the isolation media and PCR technique for their abilities to detect *B. bronchiseptica*.

MATERIALS AND METHODS

1. Determination of a transport medium

The evaluated formulations to maintain the viability of *B. bronchiseptica* for 120 hours were Amies transport medium with charcoal (DIFCO 0832-17) (23), trypticase soy broth

(TSB) (BD & Co. – 211825) (15) and phosphate buffered according to Soerensen added 5% (v/v) of bovine fetal serum (PBS+S)(29). The effect of storage temperature on the viability of *B. bronchiseptica* was also investigated. An isolate of *B. bronchiseptica*, obtained from Central Veterinary Laboratory, Weybridge, UK, was used in the experiments. Bacteria used for mixed cultures consisted of *Pasteurella multocida*, *Streptococcus suis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* obtained from the collection maintained at the “Instituto de Pesquisas Veterinárias Desidério Finamor”, Eldorado do Sul, Brazil. These bacteria were chosen because some of these genera represent the major microorganisms of the swine nasal flora (12) or because they are frequently isolated from swine nasal cavity as contaminants. Each swab was inoculated with 200µL of *B. bronchiseptica* (10^3 CFU/mL) and 4µL of mixed culture (10^2 CFU/mL), both diluted in PBS (pH 7.3). Following this, swabs were immersed in tubes containing 2mL of testing formulations and incubated at different temperatures (10°C or 27°C) and periods (24, 72 and 120 hours). A total of 180 swabs were used in the experiment, since ten repetitions of the assay were processed. Bacterial titer was determined by Miles & Misra method (21) by inoculating serial 10-fold dilutions of swab cultures in PBS (pH 7.3) onto MacConkey agar (Oxoid CM7), incubating at 37°C for 48 hours and results were expressed as colony forming units (CFU)/mL.

2. Determination of a primary isolation medium

2.1 Tested media

A new selective medium for of *B. bronchiseptica* was developed to compare with two other specific isolation media. The selectivity in this new selective medium was guaranteed by the use of the antibiotic ceftiofur, due to the specific resistance of *Bordetella* to this drug (16) and the

sensitivity of most of the bacteria present in swine nasal cavity (24). The minimal inhibitory concentration against ceftiofur reported previously for several swine bacteria (27) was the base to test five ceftiofur concentrations (0.03µg/mL, 0.25µg/mL, 0.5µg/mL, 1.0 µg/mL and 1.5 µg/mL) to apply in the new selective medium. This determination of ceftiofur concentration was assessed by an *in vitro* productivity and selectivity test previously described (18). At least ceftiofur medium was determined to contained per mL the following components: TSB (BD & Co. – 211825) 30mg, glucose 7.5mg, agar 20mg, bromothymol blue 80µg and ceftiofur 1µg.

The other isolation media tested were MacConkey agar and a modified G20G. The G20G was modified in relation to the original medium (29) in the following way: furazolidone was used instead of furaltadone and agar concentration was raised from 1.5% to 2.0%.

2.2 Animals

A total of 50 piglets with age between 50-60 days from four different nurseries presenting sneezing were sampled. The four swine farms had an effective management practices and the sows were vaccinated against atrophic rhinitis (*B. bronchiseptica* and *P. multocida* bacterin-toxoid) approximately six and two weeks prior to farrowing.

2.3 Nasal specimens and transport

Swabs of cotton fibers and aluminum shaft were introduced in previously cleaned nostrils of each piglet. And than they were transferred to Amies medium and transported at room temperature (as determined in the transport media experiment of this study) to the laboratory.

2.4 Culture procedures

All swabs were processed in laboratory before 24h. They

were streaked on MacConkey agar surface, modified G20G and ceftiofur medium (developed in this study) and all three media were incubated at 37°C for 48 hours. And the identification of *B. bronchiseptica* was based on colony morphology and biochemical characteristics, as previously proposed (19).

3. Comparison of culture and PCR for detection of *Bordetella bronchiseptica*

The same 50 nasal specimens used in the experiment of primary isolation media were submitted to PCR, after preservation into 1.5mL microtubes, containing near 1mL of own transport medium of swab tubes and stored at -20°C. Total DNA was extracted from nasal swabs using the guanidine thiocyanate method, as described elsewhere Boom et al. (2) and PCR was based in the method proposed by Hozbor *et al.* (14) with the primers Fla2- 5'-AGGCTCCAAGAGAGAAAGGCTT- 3', Fla4- 5'-TGGCGCCTGCCCTATC-3'. Amplification was performed in 25 µl containing 5 µl of DNA template, 1.5 mM MgCl₂, 200 µM each dNTP, 20 pmol of each primer, 1.0 U of Taq DNA polymerase (LGC Biotecnologia, São Paulo), 1X PCR buffer and ultra pure water. Amplified products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The 100-bp DNA ladder (LGC Biotecnologia, São Paulo) was used as a molecular size marker.

4. Statistical analysis

The percentage of recovered *B. bronchiseptica* in the transport medium determination was compared by Fisher's Test and the number of CFU/mL was analyzed by GLM procedure, following its logarithmic conversion. Mean values were compared by Tukey-Kraemer (temperature 27°C) or t-test (temperature 10°C).

No statistical analysis was made to determine which selective medium had the best performance or to compare culture to PCR detection. In both cases a simple comparison between the reached percentages by each medium (selective medium determination) or each method (culture or PCR detection) was processed.

RESULTS

Transport medium

The qualitative growth of *B. bronchiseptica* in the three transport formulations at 10°C and 27°C incubation is presented in Table 1. The recovery capability of Amies and PBS was higher than TSB during the first 72h when swabs were incubated at 10°C (P<0.05). Due to low *B. bronchiseptica* recovery with TSB formulation at 10°C in the first 72 hours, the quantitative comparison of recovery capabilities of *B. bronchiseptica* was performed only between Amies and PBS+S formulation. The number of recovered *B. bronchiseptica* cells at 24 and 72 hours was similar between Amies and PBS+S. However, at 120 hours incubation, Amies medium obtained a significantly higher bacterial count as compared to PBS+S (Table 2). Up to 72 hours, PBS+S and TSB presented no significant differences, but were superior to Amies. And at 120 hours, all the formulations differed significantly, and the highest recovery rate was obtained with TSB, followed by PBS+S and Amies (Table 2).

Selective medium

On ceftiofur medium *B. bronchiseptica* formed after 48h of incubation small (near 1.2mm diameter), convex, smooth, green to blue with darker centre, transparent colonies with a blue reaction in the medium around them. This colony morphology was quite similar to the morphology presented G20G medium, which presented superior diameter (1.5-

Table 1. Qualitative growth of *B. bronchiseptica* at 10° and 27°C

Formulation	10°C				27°C			
	24 h (%)	72 h (%)	120 h (%)	24 h (%)	72 h (%)	120 h (%)	24 h (%)	120 h (%)
Amies	8/10* (80) a	9/10 (90) a	7/10 (70)	9/10 (90)	10/10 (100)	10/10 (100)	10/10 (100)	10/10 (100)
PBS+S ¹	4/10 (40) ab	7/10 (70) a	4/10 (40)	7/10 (70)	10/10 (100)	10/10 (100)	10/10 (100)	10/10 (100)
TSB ²	2/10 (20) b	1/10 (10) b	5/10 (50)	8/10 (80)	9/10 (90)	7/10 (70)	9/10 (90)	7/10 (70)
	P<0.05	P<0.05	P=0.392	P=0.846	P=1.00	P=0.089	P=1.00	P=0.089

¹ phosphate buffer according to Soerensen added 5% of bovine fetal serum;

² trypticase soy broth;

* Number of positive results/ total number of essay repetitions;

a,b Numbers followed by different letters, in the same column, are different (P<0.05).

Table 2. Quantitative growth of *B. bronchiseptica* at 10° and 27°C

Formulation	10°C						27°C					
	24h		72h		120h		24h		72h		120h	
	10 ³ UFC/mL	Log	10 ³ UFC/mL	Log	10 ³ UFC/mL	Log	10 ⁶ UFC/mL	Log	10 ⁶ UFC/mL	Log	10 ⁶ UFC/mL	Log
Amies	0.37 ± 0.31	2.48 ± 0.28	1.43 ± 1.55	2.88 ± 0.56	6.24 ± 3.42	3.73 ± 0.26 a	0.56 ± 0.43	4.78 ± 1.40	1.75 ± 0.44	6.23 ± 0.11a	2.51 ± 1.3	6.33 ± 0.27a
PBS + S ¹	0.52 ± 0.36	2.64 ± 0.31	5.80 ± 7.24	3.46 ± 0.60	2.30 ± 3.24	2.95 ± 0.76 b	0.83 ± 1.80	5.50 ± 0.66	23.85 ± 20.47	7.25 ± 0.33b	89.90 ± 75.81	7.75 ± 0.50b
TSB ²							3.47 ± 3.74	5.80 ± 1.18	49.57 ± 38.53	7.48 ± 0.59b	584.71 ± 525.15	8.54 ± 0.53c
	P=0.390		P=0.066		P=0.031		P>0.05		P<0.0001		P<0.0001	

¹ phosphate buffer according to Soerensen added 5% of bovine fetal serum;

² trypticase soy broth;

a, b Values followed by different letters, in the same column, are different (P<0.05)

2.0mm) and inferior transparency. And the colonies on MacConkey agar were small (inferior to 1.0mm), convex, smooth, with pink hue and amber discoloration of the underlying medium.

Nine (18%) swine were positive for *B. bronchiseptica* isolation. The recovery rate of *B. bronchiseptica* was similar on MacConkey agar and modified G20G agar (14%, 7/50 samples), however, on this last medium the contaminant flora was present in higher number than MacConkey agar. The ceftiofur medium showed the worst performance, with 8% (4/50) recovery rate. And considering the three selective media together, it increased the isolation capability of each alone.

Comparison of culture and PCR results

Among sampled piglets, 26% (13/50) were positive by PCR (Figure 1.), 18% (9/50) by culture and the two techniques had 14% (7/50) of concordance in positive results. Analyzing each tested isolation medium alone, PCR agreed positively with isolation on MacConkey agar, modified G20G and ceftiofur medium, respectively, in 12%, 10% and 8% assays. PCR technique failed to detect *B. bronchiseptica* when compared to positive isolation in MacConkey, modified G20G and ceftiofur medium, respectively, in 2%, 4% and 0% assays. MacConkey agar, modified G20G and ceftiofur medium failed to isolate *B. bronchiseptica* when PCR was positive, respectively, in 14%, 16% and 18% assays.

DISCUSSION

B. bronchiseptica survived best in a transport system at 27°C and this could be explained by the nearness of this temperature to the optimal thermal condition of *B. bronchiseptica* as compared to the temperature 10°C. At

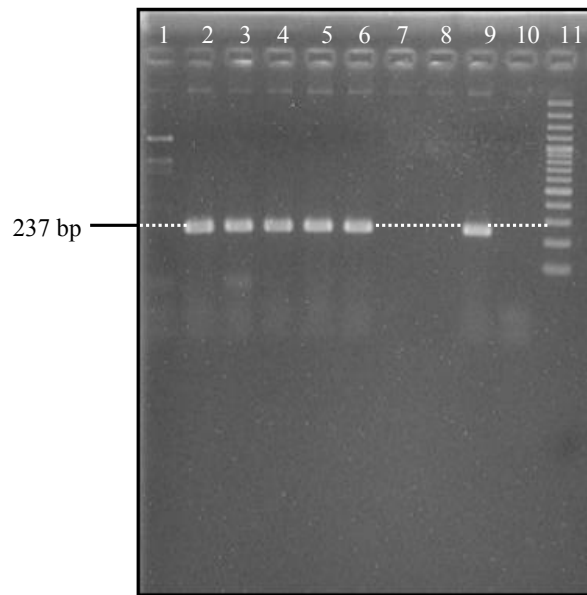


Figure 1. Amplification of a 237 bp segment of *B. bronchiseptica* DNA. Lane 2 to 6 show positive samples, lane 7 and 8 depict negative samples, lane 9 and 10 indicate the negative and positive controls and lane 11 contains molecular weight marker.

room temperature, the organism starts its multiplication and the medium then harbors greater numbers of bacteria, easing survival during transportation (13). However, some authors demonstrated that room temperature should not be recommended, since overgrowth occurs and *Bordetella* does not multiply significantly (11). This is what possibly has happened in this study, since TSB, that represents the most nutritional medium among the three tested formulations, recovered *B. bronchiseptica* in fewer opportunities than Amies and PBS+S, probably due to competition with the mixed bacteria.

At 27°C, Amies medium showed the worst quantitative performance after 72 hours and this was already expected,

since TBS has a rich nutritional composition and PBS+S was supplemented with bovine fetal serum. The number of *B. bronchiseptica* cells on Amies medium and in PBS+S formulation at 10°C remained almost stable, with a slight increase up to 120 hours. The low temperature of incubation could possibly delay microorganism growth by a decrease of enzymatic activity (1).

When *B. bronchiseptica* was recovered, independently of thermal condition, the number of bacteria was never inferior to initial inoculum, attesting the three tested formulations as good media to maintain the viability of this pathogen during its transport to laboratory. However, at 10°C incubation Amies medium or PBS+S formulation recovered in average 99% less *B. bronchiseptica* cells than at 27°C during the three recovery periods tested. Morrill *et al.* (22) also related that specimens transported at refrigerated temperature (4°C) showed suppressed overgrowth, in the case of their study the *B. pertussis* colony numbers decreased by 75%.

The ceftiofur medium showed the worst result when compared to MacConkey agar and modified G20G regarding capacity of bacterial isolation. In contrast to our results, Lariviere *et al.* (16) found better performance of a selective medium for *B. bronchiseptica* using another cephalosporin (cephalexin) as the selective agent, comparing with MacConkey agar and G20G.

PCR obtained superior capability of *B. bronchiseptica* detection to culture in all three selective media. This could be explained by the fact that PCR does not distinguish between dead and viable bacteria (13).

The disagreement between culture and positive PCR could be explained by the higher sensitivity of the molecular technique. The sensitivity of culture could be affected by factors such as low number of *B. bronchiseptica* cells in nasal cavity (asymptomatic piglets) (26), presence of damaged or

dead *B. bronchiseptica* cells before or during processing and animals previously submitted to antimicrobial therapy.

The disagreement of PCR with positive culture can be traced to several variables. Too few bacteria in nasal swabs could affect PCR sensitivity, as found in the usual protocol, which needs at least 10 organisms of *B. bronchiseptica* for detection (14). Components of swab (shafts and head fibers) could also inhibit PCR (31). In our work we collected specimens using aluminum shaft and cotton fiber head swabs, but apparently the composition had no negative influence in *B. bronchiseptica* detection, since PCR was more sensitive than culture. This finding needs to be validated, as we used just one type of swab. Other authors also did not find any inhibitory effect with swabs containing aluminum shafts (3). Some components of the transport medium, such as agar, could still affect PCR sensitivity (10). We used Amies medium and it contains agar, however it seemed not to affect the PCR, since samples containing pure culture of *B. bronchiseptica* immersed in Amies medium were previously successfully detected by PCR. DNA damage during storage could also negatively affect PCR, since the period of time between specimen collection and processing in our experiment was almost two months. Other hypothesis to explain the failure of PCR to detect *B. bronchiseptica* could be related to the presence of infection with a strain with an altered or mutated sequence in the region defined by the primers (20). Dragsted *et al.* (6) stated that the main factor that determines the quality of a PCR method is the design of primers, which should target a highly specific and preserved sequence in bacterial genome. The PCR protocol used in our study is highly specific, since the target DNA region chosen for primers design is the upstream region of the *fla* gene, a highly specific and preserved sequence in the *B. bronchiseptica* genome (14). The authors confirmed the

sensitivity and specificity of this PCR protocol testing 30 *B. bronchiseptica*, 30 *B. pertussis*, 30 *B. parapertussis* and different isolates of bacterial and fungal genera often detected in the human respiratory tract (*Aspergillus* sp., *Candida* sp., *Chlamydia* sp., *Haemophilus* sp., *Legionella* sp., *Mycoplasma* sp., *Staphylococcus* sp. and *Streptococcus* sp.).

CONCLUSIONS

Amies and PBS+S were the most efficient transport media at 27°C of incubation, however Amies medium was pointed as the preferential transport medium due to the ease of preparation.

MacConkey agar and modified G20G could be employed successfully for *B. bronchiseptica* primary isolation. Nevertheless, considering the number of recovered colonies in each medium, the ease of production and cost, MacConkey was considered the choice medium.

Culture was less sensitive than PCR. Even using the best transport temperature and medium and using the most sensitive selective medium as defined in our study.

And while the combined use of molecular and bacteriological detection has markedly increased the detection capability as compared to the use of a single method, it was shown that only part of clinically diagnosed cases could be confirmed, pointing for the possible underestimated impact of *B. bronchiseptica* infection in swine herds.

RESUMO

Desempenho de meios de transporte e seletivo na recuperação de *Bordetella bronchiseptica* de suínos e sua comparação à detecção pela reação em cadeia pela polimerase

Três ensaios comparativos foram feitos com o objetivo de aperfeiçoar a sensibilidade do diagnóstico da infecção pela *Bordetella bronchiseptica* a partir de suabes nasais de leitões. O experimento inicial comparou a recuperação de *B. bronchiseptica* a partir de suabes, simultaneamente inoculados com *B. bronchiseptica* e algumas bactérias interferentes, imersos em três formulações para transporte (meio Amies com carvão, caldo tripticaseína de soja e tampão de fosfatos segundo Soerensen suplementado com 5% de soro fetal bovino) e submetidos a diferentes temperaturas (10°C e 27°C) e períodos de incubação (24, 72 e 120 horas). O experimento subsequente comparou três meios seletivos (ágar MacConkey, meio seletivo G20G modificado e o meio de ceftiofur) em relação às suas capacidades de recuperação a partir de amostras clínicas. O último experimento comparou a reação em cadeia pela polimerase aos três meios seletivos. No primeiro experimento, a recuperação de *B. bronchiseptica* nos sistemas de transporte foi melhor a 27°C e as três formulações tiveram boas performances nesta temperatura, mas o conjunto das análises qualitativa e quantitativa apontou para o uso preferencial do meio Amies para o transporte de suabes nasais. O segundo experimento indicou que o ágar MacConkey e o G20G modificado mostraram resultados similares e foram superiores ao meio de ceftiofur. No experimento final, a técnica de reação em cadeia pela polimerase apresentou uma capacidade de detecção da *B. bronchiseptica* superior a do cultivo.

Palavras-chave: *Bordetella bronchiseptica*, suínos, meios de transporte, meios seletivos, PCR

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