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Detection of Pasteurella Multocida by qPCR Associated with Pneumonic Lung in Pigs Slaughtered in Mato Grosso Brazil

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Abstract: *Pasteurella multocida* is considered a important pathogens of swine respiratory system, causing progressive atrophic rhinitis, septicemia and pneumonia in pigs. The aim of this study is to quantify *Pasteurella multocida* in lungs of slaughtered pigs with and without pneumonia by qPCR technique and evaluate the interactions between bacteria and viruses by analyzing the association and the correlation between them using qPCR, microbiological and histopathological examinations of the lungs. Of the 109 samples collected, 61 samples showed no macroscopic lesions and 48 samples with macroscopic lesions. A total of 45.8% (22/48) of lung lesion group and 4.9% (3/61) of the group without lesions were positive for *P. multocida* isolation, being the only significant association with the lesion and control groups ($p < 0.000$). Based on the number of copies of the *kmt1* gene of *P. multocida* present in the lesion group (8.25 copies) and in the control group (1.6 copies) there is a significant difference ($p < 0.05$). The qPCR technique for detection of *P. multocida* was efficient for being more sensitive than the microbiological method, demonstrating that a larger number of *kmt1* gene copies is in GL suggest that *P. multocida* is an important agent in the development of pneumonia.

Keywords: *Pasteurella multocida*, pig, qPCR, swine respiratory complex

Introduction

Respiratory disease in pigs is a common cause of losses in modern pork production, being often referred as porcine respiratory disease complex (PRDC) (Opriessnig and others 2011). PRDC is primarily characterized as bronchopneumonia and lung lesions localized in the cranioventral parts, with consolidation areas (Hansen et al., 2010; Harms et al., 2002). Many microorganisms are associated to PRDC as bacteria *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*), *Streptococcus suis* (*S. suis*), *Haemophilus parasuis* (*H. parasuis*), *Pasteurella multocida* (*P. multocida*) (Fablet et al., 2011; Hansen et al., 2010; Pijoan, 2006), and some virus like Swine Influenza Virus (SIV), Porcine Reproductive and Respiratory Syndrome Virus (PRRS), Porcine Circovirus type-2 (PCV-2) and Porcine Respiratory Coronavirus (PRCV) (Sorensen et al., 2006).

Among them *Pasteurella multocida* is one of the most important pathogen of respiratory disease in pigs because it can be associated with progressive atrophic rhinitis, septicemia and pneumonia (Sun et al., 2010). it is considered to be a secondary pathogen of pneumonia (Pijoan, 2006; Ross, 2006). However, there are studies that it could experimentally reproduced the respiratory disease with *P. multocida.*(Ono et al., 2003). The

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detection of *P. multocida* relies mainly on qualitative techniques as bacterium isolation, serological techniques and Polymerase Chain Reaction (PCR) (Pijoan, 2006; Sun et al., 2010). The quantitative real-time PCR (qPCR) has the capability to detect and measure in a few minutes the nucleic acids of a large number of samples from numerous sources and it is an excellence technique for molecular diagnostics of life sciences, agriculture and medicine (Bustin et al., 2009).

Therefore, the aims of the present study were to quantify *P. multocida* using the qPCR and associate with the pathological and culture diagnosis from samples of pigs lungs tissues from two abattoir in Mato Grosso, Brazil.

Materials And Methods

Sampling and gross pathology

Lungs were collected during august of 2010 to march of 2011 from 109 finishing pigs, 48 lungs (GL) with pulmonary consolidation, fibrin deposition on the pleura, pleurisy and/or adhesion and 61 lungs without gross lesions (GC), from two abattoirs in Mato Grosso, Brazil. Tissue samples from the lung lesion were taken to be analyzed and the samples used as a control were taken from the right apical lobe (Hansen et al., 2010).

Microbiology and PCR

The tissue samples for the isolation of *P. multocida* were macerated and plated on 5% sheep blood agar and MacConkey agar, incubated at 37°C for 48 hours in aerobiosis, followed by the morphological and biochemical characterization (Quinn et al., 2002). PCR based on the *P. multocida* gene *kmt1* was done to confirm the isolate as previously described (Townsend et al., 2001). DNA extraction from all the lung tissue was done using phenol/choloroform method (Sambrook and Russel, 2004) to do the polymerase chain reaction (PCR) for the following microorganisms listed in Table 1.

Real-time polymerase chain reaction for Pasteurella multocida

The oligonucleotides were designed[e] from the *kmt1* gene since this gene exists in a single copy in the genome (Table 1). The PCR product was purified and cloned[a] following the manufacturer´s protocols. Each reaction is composed of supermix[b], 5 pmol of each primer, 20 ng DNA at final volume of 20µl and performed on Real-Time PCR System[c]. The reaction profile included holding stage, 95°C for 10 min., 40 cycles of denaturation, 95°C for 15 sec., annealing/extension for 60°C for 1 min., following melting curve analysis at the end of the reaction to confirm single PCR product. The detection limit or the assay was measured by testing five dilutions of the recombinant plasmid containing the *kmt1* gene of *P. multocida* (from 10^4 to 1 copy of plasmid/reaction) of a *P. multocida* strain isolated from a pig lung. The number of target copies of the tissue was calculated based on 100% DNA extraction efficiency and considering one copy of the *kmt1* gene per organism. Triplicates of the same plasmid dilution and samples were done.

Histopathology

The samples were fixed in a 10% buffered formalin for 24h, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (HE) (Allen, 1992). The sectioned tissue was evaluated as previously described (Hansen et al., 2010) and were classified as acute, subacute and chronic for the following structures: bronchi, bronchioles and bronchus-associated lymphoid tissue (BALT); alveolar ducts and alveoli, including alveolar septa; peribronchial, peribronchiolar and interlobular connective tissues and pleura.

Statistical analyses

Statistical analyses were done with the software R,[d] for association between positive pathogen test and lung lesion were analyzed by Chi square test with Pearson Uncorrected (95% confidence level).

Results

The detection of microorganism was observed in both groups, in 16.4% of the control group (CG) and 8.3% of the group lesions (GL). The detection of only one microorganism in the CG (41%) and GL (39.3%) was more frequent than the combination of more than one microorganism. *PCV-2* (21.6%) and *M. hyopneumoniae* (19.6%) were the major pathogens alone detected in the CG, however, in the GL, *P. multocida* (20.5%) was most common. The occurrence of co-infection was observed in both groups of lungs examined. In the CG, up to four microorganisms were detected, and the association of *PCV*-2 and *M. hyopneumoniae* was the most frequent. In the GL, up to five microorganisms were observed.

Regarding the occurrence of specific microorganism, a total of 45.8% of the lungs were positive for the isolation of *P. multocida* in the GL and in the CG only 4.9% of the lungs. The

detection by PCR for the following microorganisms in the GL and CG was 14.58% and 13.11% for *A. pleuropneumoniae,* 50% and 52.45% for *PCV-2*, 22.51% and 14.75% for *H. parasuis*, 6.30% and 4.90% for *S. suis,* 18.75% and 54.09% for *M. hyopneumoniae* respectively, they are listed in Table 2. Only the occurrence of *P. multocida* in lungs of CG was significant relative compared to GL ($p<0.000$). The detection of *M. hyopneumoniae,* however, drew attention because the CG had more than the double of positives when compared to GL.

The standardization of the qPCR technique was based on the standard curve and melting curve. The qPCR assay had a reaction efficiency of E = 98% ($r^2 = 0.94$), slope – 3,35 and a melting curve with a Tm of 84.4°C. In the CG 93.6% of the samples were negatives. There were 4 detections in the CG which 3 (4.9%) had up to 10 copies of the gene *kmt1*, one (1.6%) sample showed 10-100 copies of the gene *kmt1.* In the GL 50% (24/48) of the samples were negative for qPCR, 10.4% (5/48), 20.8% (10/48), and 18.8% (9/48) of the samples had less than 10 copies, 10-100 copies and more than 100 copies of the gene *kmt1* respectively. The average number of copies of the gene *kmt1* of *P. multocida* detected in the lungs of GL (8.25 copies) and CG (1.6 copies) showed a significant difference ($p<0.05$).

The gross pathology lesions observed in the GL were lung consolidation with cranial ventral distribution in 100% of the lungs (n=48). Exudates were found in bronchi and bronchioles of the GL, seromucous was more frequent with 37% (18/48) of the lungs, following mucopurulent 8,3% (4/48) and 2% (1/48). Presence of adhesion of the parietal and visceral pleura occurred in 31% of the lungs. In the CG there were no presence of exudates in the bronchi and bronchioles observed.

Based on the histopathological characterization (table 3) observed, the lungs in the CG did not have any histological characterization (98.36%). BALT hyperplasia was observed in 3 (5%) samples. In the CG 12 samples (25%) did not have bronchopneumonia lesions (BP), but the BALT hyperplasia was detected in most of them (10/12). Among the lungs with pneumonia, the most prevalent was acute cases (n=14) with suppurative exudates (ASBP), followed by subacute cases (n=13), subdivided into BP mixed (SMBP) (n=6) and suppurative (SSBP) (n=7). The proliferation of BALT occurred frequently in acute, subacute and chronic cases. Others histopathological characterizations were found but in smaller numbers, such as interstitial edema, presence of fibrin and necrosis.

Association between isolation and detection by PCR with microscopic lesions were observe in the both groups. In the CG, we observed the presence of microorganism in various lungs, however the presence of microscopic lesions of NSBP is associated with detection of *M. hyopneumoniae* and *H. parasuis.* In GL the detection of *P. multocida* was the most frequent in ASBP (50%), SMBP (67%), SNBP (57%) and CSBP (56%). The *PCV-2* was also very often being detected in SMBP (67%) and CSBP (56%.). There was no significant association observed between microorganisms detected and a specific pattern of histological changes in lungs. In qPCR of *P. multocida*, average numbers of copies of *kmt1* gene in acute (21), subacute (50) and chronic (180.9) pattern were different but only significant statistically between acute and chronic cases.

Discussion

Most authors define PRDC as multifactorial respiratory disease involving several pathogens, bacteria and viruses (Hansen et al., 2010; Harms et al., 2002; Opriessnig et al., 2011). In the present study we analyzes the importance of *P. multocida* and the presence of major pathogens causing pneumonia (*PCV-2, A. pleuropneumoniae, M. hyopneumoniae, H. parasuis* and *S. suis*) with this pathogen, known to increase respiratory lesions of pneumonia in pigs slaughtered in Mato Grosso state. This disease cause economic losses for condemnation of carcasses and organs, medicines and vaccines, as well as lower development of the animal in the production system.

Microbiological analyzes performed on 109 samples (CG and GL) showed that 22 (20.1%) were negative for all pathogens tested. In previously studies in Denmark(Hansen and others 2010) 9.45% (14/148) samples were negative for any *Mycoplasma* spp. bacteria and respiratory viruses. The percentage of negative lungs in the GL may be due to the presence of other pathogens that can also cause pneumonia in swine, for example: SIV, PRRSV, PRCV, and *M. hyorhinis,* (Hansen et al., 2010; Sorensen et al., 2006) the presence of mycotoxins or elimination of the pathogen in chronic infections (Hansen et al., 2010).

Studies similar to that performed with lung fragments in Denmark (Hansen et al., 2010) and Germany, (Palzer et al., 2008) with bronchoalveolar lavage, detected the same pathogens in this study. In the study of Hansen (2010) *P. multocida* was significant for the

development of pneumonia in pigs, which agrees with these results. However, the work of Palzer (2008) the presence of *P. multocida* was not statistically significant for development of pneumonia, but the presence of α-hemolytic *Streptococcus* was, irrespective of the species of *Streptococcus*. In the present study the detection of *S. suis* was done, but was not significant and occurred with low frequency.

PCV-2 was detected in over 50% of samples in both groups, suggesting that the virus is circulating in swine, but there was no statistically significant difference between groups.

The co-infections have been described in several studies, (Fablet et al., 2011; Hansen et al., 2010; Opriessnig et al., 2011; Pors et al., 2011) in this study the co-infection more common was *P. multocida* and *PCV-2* in GL, which also was seen in studies of Hansen (2010). In the CG the co-infection most frequent was *PCV-2* and *M. hyopneumoniae*, which in the study of Hansen (2010) was different, the association found was *PCV*-2 and *S. suis*, and on work of Palzer (2008), the most significant association among others were *P. multocida* and *Bordetella bronchiseptica*, α-hemolytic *Streptococcus* and *H. parasuis*, *M. hyopneumoniae* and *PCV-2*.

Gross and histopathological lesions along with microbiological analyzes from lungs with and without lesions were used to detect which main pathogens causing pneumonia were involved with the bacterium *P. multocida* known to increase respiratory lesions (Fablet et al., 2011; Hansen et al., 2010; Pijoan, 2006). However, this study showed that in several lungs only *P. multocida* was detected, without associated pathogens that could act synergistically as *PCV-2* and *M. hyopneumoniae* (Hansen et al., 2010).

The acute lesions were more frequent in the lungs of GL (29.6%), which contrasts with the study by Hansen (2010) in which 77% of the lesions were chronic. In our study, probably the stress caused by transport over long distances of animals to the slaughterhouse, or due to withdrawal of antibiotics used in the finishing phase had increased susceptibility to agents.

In this study the histopathological characterization found differ from Hansen (2010), in which the CG, all samples were characterized as no microscopic lesions, whereas in the present study there was a sample which was characterized by the presence of an acute injury, which may indicate the early stage of pneumonia. In both studies the CG presented a percentage of samples with BALT hyperplasia, about 17% in this study, this is mainly due to the high occurrence of *M. hyopneumoniae* (53%) in the GC that induces an infiltration of inflammatory cells and stimulation of BALT (Opriessnig et al., 2004).

Among the association of microorganisms and the type of lesion found, the presence of *P. multocida* in both groups was significant, highlighting the presence of the pathogen in the following types of injuries: ASBP, SMBP, SNBP and CSBP. The associations of other pathogens and histological patterns of bronchopneumonia were not significant as described by Hansen (2010).

Quantification of microorganisms has been used to analyze correlations between the microbial and tissue lesions or clinical signs. The use of qPCR for the *kmt1* gene demonstrated a difference between the number of copies relative to the CG and GL, which could be used as a tool for differentiating animals bearing from sick animals. Regarding the histological patterns of BP acute, subacute and chronic non-significant differences were observed, however a larger sample size is needed for a definitive analysis.

The qPCR technique for detection of P. multocida was efficient for being more sensitive than the microbiological method, showing that the largest number of copies of the gene *kmt1* is in GL. This result suggests that *P. multocida* is an important agent in the development of pneumonia.

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Soucers And Manufactures

a. CloneJET™ PCR Cloning Kit, Fermentas, Madson, WI, USA.

b. Platinum SYBR Green qPCR Supermix, Invitrogen, Carlsbad, CA, USA.

c. Applied Biosystem StepOne™ Real-Time PCR System, Foster City, CA, USA.

d. R: Development Core Team

e. NCBI/ Primer-BLAST tool

References

ALLEN, T. C. (1992) Hematoxylin and eosin. In Laboratory methods in histotechnology. Ed E. B. PROPHET. Washington D. C, American Registry of Pathology. pp 53-58

ANGEN, O., OLIVEIRA, S., AHRENS, P., SVENSMARK, B. & LESER, T. D. (2007) Development of an improved species specific PCR test for detection of Haemophilus

parasuis. Vet Microbiol 119, 266-276

BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55, 611-622

CHATELLIER, S., HAREL, J., ZHANG, Y., GOTTSCHALK, M., HIGGINS, R., DEVRIESE, L. A. & BROUSSEAU, R. (1998) Phylogenetic diversity of Streptococcus suis strains of various serotypes as revealed by 16S rRNA gene sequence comparison. Int J Syst Bacteriol 48 Pt 2, 581-589

FABLET, C., MAROIS, C., DORENLOR, V., EONO, F., EVENO, E., JOLLY, J. P., LE DEVENDEC, L., KOBISCH, M., MADEC, F. & ROSE, N. (2011) Bacterial pathogens associated with lung lesions in slaughter pigs from 125 herds. Res Vet Sci

FENAUX, M., HALBUR, P. G., GILL, M., TOTH, T. E. & MENG, X. J. (2000) Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J Clin Microbiol 38, 2494-2503

HANSEN, M. S., PORS, S. E., JENSEN, H. E., BILLE-HANSEN, V., BISGAARD, M., FLACHS, E. M. & NIELSEN, O. L. (2010) An Investigation of the Pathology and Pathogens Associated with Porcine Respiratory Disease Complex in Denmark. Journal of Comparative Pathology 143, 120-131

HARMS, P. A., HALBUR, P. G. & SORDEN, S. D. (2002) Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. Journal of Swine Health and Production 10, 27-30

MATTSSON, J. G., BERGSTROM, K., WALLGREN, P. & JOHANSSON, K. E. (1995) Detection of Mycoplasma hyopneumoniae in nose swabs from pigs by in vitro amplification of the 16S rRNA gene. Journal of Clinical Microbiology 33, 893-897

ONO, M., OKADA, M., NAMIMATSU, T., FUJII, S., MUKAI, T. & SAKANO, T. (2003) Septicaemia and Arthritis in Pigs Experimentally Infected with Pasteurella multocida Capsular Serotype A. Journal of Comparative Pathology 129, 251-258

OPRIESSNIG, T., GIMÉNEZ-LIROLA, L. G. & HALBUR, P. G. (2011) Polymicrobial respiratory disease in pigs. Anim Health Res Rev 12, 133-148
OPRIESSNIG, T., THACKER, E., YU, S., FENAUX, M., MENG, X. & HALBUR, P. (2004) Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneamoniae and porcine circovirus type 2. Veterinary Pathology 41, 624-640

PALZER, A., RITZMANN, M., WOLF, G. & HEINRITZI, K. (2008) Associations between pathogens in healthy pigs and pigs with pneumonia. Veterinary Record 162, 267-271

PIJOAN, C. (2006) Pneumonic Pasteurellosis. In Disease of Swine. 9 edn. Ed B. E. E. A. E. STRAW. Iowa, Iowa State University Press. pp 719-726

PORS, S. E., HANSEN, M. S., CHRISTENSEN, H., JENSEN, H. E., PETERSEN, A. & BISGAARD, M. (2011) Genetic diversity and associated pathology of Pasteurella multocida isolated from porcine pneumonia. Vet Microbiol 150, 354-361

QUINN, P. J., MARKEY, B. K., CARTER, M. E., DONNELLY, W. J. & LEONARD, F. C. (2002) Veterinary Microbiology and Microbial Disease, Blackwell Science Ltd
ROSS, R. F. (2006) Pasteurella multocida and its role in porcine pneumonia. Animal Health Research Reviews 7, 13-29

SAMBROOK, J. & RUSSEL, D. W. (2004) Molecular cloning A laboratory Manual. New York, Cold Spring Harbor laboratory Press

SORENSEN, V., JORSAL, S. E. & MOUSING, J. (2006) Diseases of the respiratory system. In Diseases of swine. 9th edn. Ed B. STRAW, ZIMMERMANN, W., D'ALLAIRE, S., TAYLOR, D.J. Ames, Iowa State University Press. pp 149-177

SUN, D., WANG, J., WU, R., WANG, C., HE, X., ZHENG, J. & YANG, H. (2010) Development of a novel LAMP diagnostic method for visible detection of swine Pasteurella multocida. Veterinary Research Communications 34, 649-657

TOWNSEND, K. M., BOYCE, J. D., CHUNG, J. Y., FROST, A. J. & ADLER, B. (2001) Genetic organization of Pasteurella multocida cap Loci and development of a multiplex capsular PCR typing system. J Clin Microbiol 39, 924-929

XIAO, G. S., CAO, S. J., DUAN, L. L., WEN, X. T., MA, X. P. & CHEN, H. M. (2006) Identification and Detection of Actinobacillus pleuropneumoniae in Infected and Subclinically Infected Pigs by Multiplex PCR Based on the Genes ApxIVA and OmlA. Agricultural Sciences in China 5, 146-154

Tabel 1. Oligonucleotide sequence of primers for the PCR and qPCR.

Microorganism	Target gene	Name	Sequence (5' to 3')	pb*
Actinobacillus pleuropneumoniae	Om1A	apxIVF† apxIVR	ATACGGTTAATGGCGGTAATGG ACCTGAGTGCTCACCAACG	346
PCV-2	ORF2	PCV2 SEM‡ PCV2 ANT	CACGGATATTGTAGTCCTGGT CCGCACCGGATATACTGTC	494
Haemophillus parasuis	16S rDNA	HP1F3§ HP2F2 Revx	TATCGRGAGATGAAAGAC GTAATGTCTAAGGACTAG CCTGGCTTCGTC	1090
Streptococcus suis	16S rRNA	16S195(s)¦ 16S489(as2)	CAGTATTTACCGCATGGTAGATAT GTAAGATACCGTCAAGTGAGAA	294
Mycoplasma hyopneumoniae	16S rRNA	Forward# Reverse	GAGCCTTCAAGCTTCACCAAGA TGRGRRAGRGACTTTTGCCACC	649
P. multocida (qPCR)	*Kmt1*	Forward Reverse	CACTTGAAATGGGAAATTCG TTGTCAAGGAAGCAGATTGG	121

*Base pairs; †(Xiao et al., 2006); ‡(Fenaux et al., 2000); §(Angen et al., 2007); ¦(Chatellier et al., 1998); #(Mattsson et al., 1995);

Table 2. Association between infectious pathogens and patterns of pneumonia in pigs slaughtered in accordance with the degree of lesion.

NSBP: supurative bronchopneumonia in control group ; ASBP: supurative broncopneumonia in acute group lesion;

	Control Group				Group Lesion							
			Acute		Acute		Subacute				Chronic	
	Normal (n=60)		NSBP (n=1)		ASBP (n=14)		SMBP (n=6)		SNBP (n=7)		CSBP (n=9)	
	n	%	n	%	n	%	n	%	n	%	n	%
P. multocida	3	5	0	-	7	50	4	67	4	57	5	56
A. pleuropneumoniae	8	13	0	-	2	14	0	-	2	29	2	22
PCV-2	32	53	0	-	5	36	4	67	3	43	5	56
H. parasuis	8	13	1	100	3	21	0	-	1	14	4	44
M. hyopneumonie	32	53	1	100	5	36	2	33	0	-	2	22
S.suis	3	5	0	-	2	14	0	-	0	-	1	11

SMBP: mixed bronchopneumonia in subacute in group lesion; SNBP: nonsuppurative bronchopneumonia in subacut in group lesion; CSBP: suppurative bronchopneumoniae in chronic in group lesion

Table 3. Histopathological findings in lungs of pigs CG and GL

	Control				Case group							
	Normal (n=60)		Acute (n=1)		Normal (n=12)		Acute (n=14)		Subacute (n=13)		Chronic (n=9)	
	n	%	n	%	n	%	n	%	n	%	n	%
BALT hyperplasia*												
0	51	85	0	-	3	25	2	14	1	8	1	11
+	3	5	1	100	7	58	6	42	8	62	3	33
+ +	0	-	0	-	1	8	6	42	4	31	3	33
+ + +	0	-	0	-	1	8	0	-	0	-	2	22
+ + + +	0	-	0	-	0	-	0	-	0	-	0	-
Atelectasis	0	-	0	-	1	8	0	-	0	-	0	-
Alveolar Hyperplasia	0	-	0	-	1	8	0	-	0	-	0	-
Intersticial oedema	0	-	0	-	0	-	5	36	1	8	1	11
Necrotic foci	0	-	0	-	0	-	0	-	0	-	1	11

* Hyperplasia of the BALT wass scored as absent (0), mild (+), moderate (+ +), marked (+ + +) or extensive (+ + + +)