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Occurrence and associated lesions of *Pasteurella multocida* in porcine bronchopneumonia

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Abstract

With the aim to extend the present knowledge on possible systemic spreading of *P. multocida* in pigs with bronchopneumonia, the occurrence and associated lesions of *P. multocida* was described by comparing cultural detection, pathological evaluation and *in situ* hybridization of *P. multocida* in lungs, hearts and kidneys from cases of porcine bronchopneumonia. *P. multocida* was cultivated from the lung lesions in 114 out of a total of 148 cases of porcine bronchopneumonia. Among the 114 cases, *P. multocida* was also cultivated from the pericardial sacs of 40 pigs and the kidneys of seven pigs. Gross lesions and histological findings included a variety of type and stages of bronchopneumonia in connection to the isolation of *P. multocida*. Furthermore, chronic fibrous pericarditis, interstitial nephritis and a high proportion of lympho-histocytic nephritis were observed. *In situ* hybridization identified *P. multocida* in the majority of the lungs, none of the

hearts and in half of the kidneys examined. The results show a possible low rate of systemic spreading of *P. multocida* from lung lesions in pigs with bronchopneumonia.

Keywords: *Pasteurella multocida*, *in situ* hybridization, pigs, bronchopneumonia, sepsis

Introduction

Infectious porcine bronchopneumonia is a widespread disease of major economic significance (Sørensen et al. 2006). In a Danish study, bronchopneumonia was present in 25% of the pigs at slaughter (Christensen & Enoe 1999) and a similar frequency has been reported from other countries (von Altrock 1998; Maes et al. 2001). A frequent finding among the infectious agents associated with bronchopneumonia in pigs is the Gram-negative bacterium *Pasteurella multocida* (Gois et al. 1975; Morrison et al. 1985; Falk et al. 1991; von Altrock 1998; Ross 2006; Hansen et al. 2010). However, *P. multocida* is rarely considered the primary agent, but rather constitutes a part of the porcine respiratory disease complex (PRDC) (Falk et al. 1991; Thacker 2001; Ross 2006; Hansen et al. 2010). It is generally accepted, that *P. multocida* plays an important role by aggravating lung lesions, but little is known about the mechanisms behind, including the host response (Amass et al. 1994; Pijoan 2006; Ross 2006; Hansen et al. 2010). Lesions associated with *P. multocida* are usually characterized by exudation into bronchi and bronchioles, but necrosis of lung tissue and sepsis may also be observed (Roberts et al. 1962; Pijoan & Fuentes 1987; Mackie et al. 1992; Cameron et al. 1996; Pijoan 2006). In addition, characterization and comparison of isolates of *P. multocida* from porcine lungs and internal organs thus indicate a systemic spreading of the bacterium may take place (Buttenschon & Rosendal 1990; Buttenschon 1991; Lainson et al. 2002). Indications of systemic spreading of *P. multocida* have so far mainly been based on cultivation. In a few experimental studies and a single study on natural infections, *P. multocida* was

identified in the lesions by immunohistochemistry (Thomson et al. 2001; Ono et al. 2003) or *in situ* hybridization (Mbuthia et al. 2001). Experimental studies of *P. multocida* bronchopneumonia have, however, not provided clear results as to the risk of haematogenous spreading (Bentley & Farrington 1980; Pijoan & Fuentes 1987; Hall et al. 1990; Ono et al. 2003). The possibility of dissemination, in combination with the fact that *P. multocida* represents a zoonosis (Bisgaard et al. 1994; Iaria & Cascio 2007), indicates that further studies on the possibility and nature of dissemination are important for food safety reasons. With the aim to extend the present knowledge on possible systemic spreading of *P. multocida* in pigs with bronchopneumonia, the occurrence and associated lesions of *P. multocida* was described by comparing cultural detection, pathological evaluation and *in situ* hybridization of *P. multocida* in lungs, hearts and kidneys from cases of porcine bronchopneumonia.

Materials and methods

Sampling, gross pathology and bacteriology

Lungs, heart, kidneys and spleen were obtained from a total of 148 pigs suffering from cranioventrally located bronchopneumonia, and 60 pigs without gross lesions (controls). The sampling of both cases and controls was done simultaneous and random at two Danish abattoirs (Hansen et al., 2010). All organs were evaluated macroscopically and the type, extent and estimated duration of the lesions were recorded. Due to meat inspection procedures it was necessary to open the heart at the slaughterhouse. Therefore, the pericardial sac was cultured immediately after sampling by aseptically applying a sterile cotton swab on the surface of the heart (epicardium) as close to the heart basis as possible. The pericardium was separated from the surface of the heart (epicardium) if fibrotic fibrous pericarditis was present. The remaining samples were kept cooled and processed within 5 hours (Hansen et al., 2010). Lungs, kidneys and spleen were cultured

bacteriologically using sterile cotton swabs after heat decontamination on the surface of gross lesions, if such were present. The swabs were plated on blood agar (Blood agar Base CM 55, Oxoid, Basingstoke, United Kingdom, added 5% bovine blood) and incubated at 37°C in a sealed plastic bag for 24 h. All samples were cross-inoculated with a v-factor producing *Acinetobacter calcoaeticus*. Isolates showing typical colony morphology of *P. multocida* were sub-cultured and identified using standard methods for phenotypic characterization. Presence of *P. multocida* was evaluated semi-quantitatively according to the number of bacterial colonies on the primary plate: weak growth (1-25 colonies), dense growth (more than 25 but less than 300 colonies) and massive growth (>300 colonies).

Histopathology

From lung, heart (epicardium), kidney and spleen, tissue specimens were obtained for histology. The specimens were fixed in 10 % neutral buffered formalin for 24 h, processed by standard histological procedures through graded concentrations of ethanol and xylene, and finally embedded in paraffin wax. From pigs, in which a positive cultivation of *P. multocida* from the lungs was obtained, the tissue samples of lung, heart and kidney were sectioned 3-5 µm thick, stained with haematoxylin and eosin (Stevens & Wilson 1996), and evaluated by light microscopy (Olympus BX45). Furthermore, the same organs from 20 pigs without lung lesions were sectioned. These were randomly selected among the 60 controls after excluding pigs with positive cultivation of *P. multocida* from the lung. Based on histological lung lesions, cases were divided into groups of bronchopneumonia as defined by Hansen *et al.* (2010). However, minor modifications in the classification of histological lesions were performed and a group of chronic necrotizing bronchopneumonia was defined. Furthermore, subacute cases were considered as one group regardless of exudate present. These modifications lead to the following groups of histopathology:

95 acute, subacute, chronic necrotizing, chronic suppurative, chronic mixed and chronic non-
 96 suppurative bronchopneumonia.

97

98 *In situ hybridization*

99 From pigs culturing positive for *P. multocida* in lung lesions and the pericardial sac or the kidney,
 100 specific *in situ* hybridization was carried out on tissue sections from lung, heart and kidney.
 101 Additionally, corresponding tissue sections of five randomly selected pigs, from which *P. multocida*
 102 was only recovered from the lungs, were processed, too. Five control pigs without lung lesions and
 103 sampling negative for *P. multocida* were included. A cyanine-3 (Cy3) labelled oligonucleotide
 104 probe, specific for the 16S rRNA of *P. multocida* (Mbuthia et al. 2001), with the sequence, 5'-
 105 CTATTTAACAACATCCCTTC-3' (TagC, Copenhagen, Denmark), was used for detection of *P.*
 106 *multocida*. A Cy3 labelled nonsense probe, non-EUB338 (5'- CGACGGAGGGGCATCCTCA-3',
 107 TagC), complementary to the universal bacterial probe, EUB338 (Amann et al., 1990), served as a
 108 negative control for nonspecific binding. All *in situ* hybridizations were run in parallel with sections
 109 of experimentally infected murine lung tissue which served as internal positive controls for the
 110 quality of hybridization. All sections were mounted on Super Frost®Plus slides (Mensel-Gläser,
 111 Braunschweig, Germany) and heated at 60°C for 1 h followed by de-paraffinization, including two
 112 changes in xylene and rehydration twice in 99.9 % ethanol for 3 min. After air drying, a circle was
 113 drawn around the tissue section using a hydrophobic pen (PAP-pen, Dakocytomation, Glostrup,
 114 Denmark) and 100 µl of hybridization buffer (100mM Tris [pH 7.2], 0.9M NaCl, 0.1% sodium
 115 dodecyl sulfate) with 500 ng probe was applied to the sections followed by incubation for 18 h at
 116 45°C. Subsequently, the sections were washed in pre-warmed hybridization buffer for 15 min at
 117 45°C in the dark and washed in pre-warmed washing buffer (100mM Tris [pH 7.2], 0.9M NaCl) for
 118 15 min, rinsed in water and mounted with DAKO mounting medium (Dakocytomation). For

detection of fluorescence, a microscope (AxioImager M1, Carl Zeiss) equipped for fluorescence with a 100-W Halogen lamp and filter sets for visualization of Cy3, was used.

Statistical analysis

Associations between lesions and cultivation of *P. multocida* were determined using Fisher's exact test. Agreement between results of cultivation and *in situ* hybridization was determined by McNemar's exact test. All analyses were done in SAS version 9.1 (SAS Institute, Inc., Cary, NC) with a statistical significant level set at $P < 0.05$.

Results

Specimens and bacteriology

P. multocida was isolated from the lungs of 114 pigs with bronchopneumonia of these 85 % (97/114) were in pure cultures (Table 1). *P. multocida* was also cultivated from the pericardial sac in 40 of the cases and 73 % (11/40) of these were in pure cultures. Furthermore, *P. multocida* was isolated from the kidney in seven cases and these were all pure cultures. *P. multocida* was found in dense or massive growth in a significantly higher frequency from the lungs compared to the cultivations from pericardial sac and kidney ($P < 0.001$) (Table 1). Among the controls, *P. multocida* was cultivated in mixed culture from the lungs of three pigs, in mixed culture from the pericardial sac of one animal and none of the kidneys. *P. multocida* was not isolated from spleens in cases or controls. In total the 114 cases and 60 controls represented 121 different swine herds.

Gross pathology

The lesions in the 114 lungs from which *P. multocida* was cultured, included acute, subacute and chronic cases (Table 1). In acute cases, the affected lung tissue was oedematous, hyperaemic and

elevated above the level of the unaffected tissue. Chronic cases were characterized by marked dilation of bronchi and bronchioles, surrounded by a rim of firm and dark-red lung tissue. Furthermore, the pneumonic areas were clearly atelectatic compared to the bordering normal lung tissue. The subacute cases were in level with the surrounding lung tissue, and the dilation of the bronchioles was diminished. In all cases, the bronchopneumonic areas were sharply demarcated from the bordering normal lung tissue. Two distinguishable types of gross lesions were found i.e. exudative or necrotizing. Exudative lesions were characterized by muco-purulent to purulent exudation into the air conducting pathways. The necrotizing type was seen in a single subacute case and eight cases of chronic lesions. In these lesions, dry foci of lung tissue with demarcation from the surrounding tissue were seen in addition to muco-purulent to purulent exudation.

In 18 of the 114 pigs, with bronchopneumonia and positive culture of *P. multocida* from the lungs, diffuse fibrotic pericarditis was observed. Four of these had positive cultivation of *P. multocida* from the pericardial sac. In 13 of the 114 cases kidney lesions were present and these were chronic, multifocal, white areas protruding through cortex and medulla corresponding to interstitial nephritis. Two of these had positive cultivation of *P. multocida* from the kidney. The extra-pulmonary lesions in the heart and kidneys were only observed in pigs with subacute or chronic bronchopneumonia. No significant difference in the occurrence of extra-pulmonary lesions was found between the subacute and chronic cases ($P=0.4$). In addition, the occurrence of gross extra-pulmonary lesions was not associated with the type of bronchopneumonia from which *P. multocida* had been isolated ($P=0.5$). Isolation of the *P. multocida* from the pericardial sac and/or kidney was not associated with presence of gross lesions found in these organs. Furthermore, the isolation of the *P. multocida* from the pericardial sac and/or kidney was not associated with any stage ($P=0.49$) or type ($P=0.45$) of the gross lung lesions found.

167

168 *Histopathology*

169 The histomorphology of lung lesions which were observed on the gross level as necrotizing were
170 characterized by multi-focal necrosis. In the necrotic centres reminiscences of pulmonary structures
171 could sometimes be observed (coagulation necrosis), while in other cases the centres were made up
172 by a homogeneous mass. Furthermore, mineralization of necrotic tissue was sometimes present. The
173 necrotic lung tissue was encircled by a zone of neutrophils. In some cases oat-shaped granulocytes
174 were present, too. The zone of neutrophils was surrounded by a fibrous capsule (Fig. 1A). In lungs,
175 with gross lesions of exudative bronchopneumonia, the lung tissue was intact and a marked
176 consolidation was evident. Lungs with this type of pneumonia were divided into three groups
177 according to the type of exudate in alveoli (suppurative, mixed and non-suppurative) (Table 2). In
178 suppurative cases the alveoli and bronchioles were filled with a mixture of inflammatory cells
179 dominated by neutrophils (Fig. 1B), while in cases of mixed bronchopneumonia, a mixture of
180 inflammatory cells including neutrophils, macrophages and other mononuclear cells were found. In
181 the non-suppurative cases, the exudate in alveoli was dominated by mononuclear cells. There was
182 not a significant difference in the frequency of cultural detection of *P. multocida* from the
183 pericardial sac and/or kidney between the groups of lung histopathology ($P=0.25$). Pericarditis,
184 corresponding to the macroscopic findings of epicardial fibrosis (Fig. 1C) and a single case of
185 lymphocytic infiltration of the myocardium was found (Table 2).

186

187 Lesions were present in nearly all sections of kidneys examined from both the pigs with
188 bronchopneumonia (81%) and in the controls (90%). The lesions were characterized as multifocal,
189 interstitial lympho-histiocytic nephritis located peritubular, periglomerular and perivascular
190 accompanied by a light fibrosis. Often the lesions were accompanied by primary lymph follicle

formation (follicular nephritis) (Fig. 1D). There was no differences in the frequency of histological lesions found in the heart and kidneys of the pigs culturing positive for *P. multocida* from the kidney and/or pericardium, compared to the pigs from which *P. multocida* was only isolated from the lungs (heart; $P=0.43$; kidney; $P=0.63$) or compared to pigs with out lung lesions (controls) (heart; $P=0.82$; kidney; $P=0.27$).

In situ hybridization

In situ hybridization demonstrated *P. multocida* in the lungs of 41 out of 47 examined cases of bronchopneumonia from which *P. multocida* was isolated (Table 3). The six lung samples found negative by *in situ* hybridization was from cases with weak growth of *P. multocida* on the primary plates. The localization of *P. multocida* in the lung tissue differed according to the types of lesions. In cases with necrosis (Fig. 1E) the bacteria were concentrated in the areas with necrotic tissue, whereas in exudative lesions (suppurative, mixed and non-suppurative) they were located mainly within alveoli and bronchioles (Fig. 1F). *P. multocida* was not demonstrated in tissue samples from the heart of any pigs, while *P. multocida* was identified within lesions of two kidneys. Additionally, three kidneys showed positive *in situ* signals in areas without visible lesions (Table 3). Cultural detection of *P. multocida* agreed with detection by *in situ* hybridization in the lung (McNemars test, $P=0.06$) and kidney (McNemars test, $P=0.7$), but not in the heart (McNemars test, $P<0.0001$).

Discussion

In the present study dissemination of *P. multocida* was evaluated in natural cases of porcine *P. multocida* pneumonia by comparing pathology with cultural and *in situ* detection of *P. multocida*. Using a specific probe for detection by *in situ* hybridization, allows detection of specific bacteria within the lesion sites (Moter & Gobel 2000). However, the method provides the presence of a

215 certain level of rRNA in the bacterium and therefore is dependent on the functional state of the
 216 bacterium (Amann et al. 1995; Moter & Gobel 2000). Cultivation represents a more sensitive
 217 method for description of the bacterial flora present in the tissues, but it does not provide
 218 information about the distribution and thereby the importance of the bacterium in the lesions
 219 (Amann et al. 1995). Combining these methods with a pathological description forms a stronger
 220 basis for evaluating the significance of the cultivated bacteria. *P. multocida* is generally recognized
 221 as a secondary invader contributing substantially to respiratory diseases in pigs by aggravation of
 222 lung lesions (Falk et al. 1991; Pijoan 2006; Hansen et al. 2010). Systemic spreading of *P.*
 223 *multocida* capsular serotype A from lung lesions has been reported in natural infections by
 224 Buttenschon (1991) and Pijoan and Fuentes (1987). Likewise in experimental infection a systemic
 225 spread of the bacterium has been reported (Ono et al. 2003). Furthermore, genetic identical isolates
 226 of *P. multocida* obtained from lungs and kidneys have been demonstrated (Buttenschon & Rosendal
 227 1990; Lainson et al. 2002). In the present study all cases examined demonstrated lesions in the
 228 lungs comparable to previous studies of *P. multocida* pneumonia (Roberts et al. 1962; Pijoan 2006)
 229 and the majority of the lungs examined by *in situ* hybridization also demonstrated positive
 230 identification of *P. multocida*, underlining the importance of this organism in porcine
 231 bronchopneumonia. No association between the character of lesions found in lung, kidney and heart
 232 was found and no association between lesions in the lung and the cultivation of *P. multocida* from
 233 pericardial sac and/or kidney was found. This indicates that spreading of *P. multocida* from the lung
 234 lesions, if it takes place, is independent of the character of lesions found in the lung.
 235 Pericarditis has previously been found in association with porcine bronchopneumonia (Buttenschon
 236 1991; Christensen & Enoe 1999), but a high frequency of cultivation of *P. multocida* from the
 237 pericardial sac has not been reported (Buttenschon et al. 1997). In this study pericarditis was found
 238 in 18 out of the 114 cases of bronchopneumonia with positive cultivation of *P. multocida*.

239 Furthermore, *P. multocida* was cultivated from the pericardial sac in 40 of the cases. However, the
240 cultivation was not associated with gross or histological lesions. The findings of pericarditis and
241 positive cultivation of *P. multocida* from the pericardial sac indicate that bronchopneumonia
242 associated with *P. multocida* is a part of a more complex disease pattern affecting other organ
243 systems in addition to the respiratory tract. In the study by Buttenschon (1991), a connection
244 between bronchopneumonia and cases of focal or diffuse nephritis at the gross level was observed.
245 Furthermore, a high incidence of isolation of *P. multocida* was found in both bronchopneumonia
246 (29 of 49 cases) and nephritis (19 of 49 cases) (Buttenschon & Rosendal 1990; Buttenschon 1991).
247 In the present study, a high incidence of bronchopneumonia concurrent with gross lesions in
248 kidneys was not observed. Furthermore, only seven isolates of *P. multocida*, all in pure culture,
249 were obtained from kidneys from cases of porcine bronchopneumonia with positive cultivation of
250 *P. multocida*. However, nearly all kidneys examined by histopathology had lesions, regardless of
251 the cultivation of *P. multocida* from the kidneys. The lesions included multifocal interstitial
252 lympho-histiocytic nephritis located peritubular, periglomerular and perivascular, accompanied by
253 light fibrosis and lympho-nodular hyperplasia. These findings differ from what was reported by
254 Buttenschon (1991), who described the lesions found in the kidney as granulomatous nephritis and
255 fibrosis. Moreover, it does not correspond to reports of acute lesions in kidneys due to *P. multocida*
256 infections (Thomson et al. 2001; Ono et al. 2003). These differences may be due to variation in time
257 span of the infections observed. *P. multocida* is considered part of the porcine respiratory disease
258 complex (PRDC) and therefore, also other pathogens present in the host could contribute. The
259 lesions described in the present study have previously been associated with viral infections such as
260 Porcine Circovirus type 2 (PCV2) and Porcine Parvovirus (Smith 1973; Drolet et al. 2002; Martinez
261 et al. 2006). PCV2 was detected in all pigs used in this study (Hansen *et al.*, 2010) and could
262 therefore be a possible cause to the lesions found in the kidneys. Furthermore, the host general

immune status and the environmental factors can influence the outcome of infection and thereby the development of lesions and possible systemic spread of *P. multocida*.

Isolates of *P. multocida* obtained from the cases described in the present study have subsequently been characterized by pulsed-field gel electrophoresis (Pors et al., unpublished results) which showed that for nine out of 24 cases, the isolates obtained from lung, pericardium and/or kidney were identical. Similar results have been reported by Buttenschon and Rosendal (1990) and Lainson et al (2002). These findings, together with the results in the present study, indicate that spreading of the infection is possible. In conclusion, a low rate of systemic spreading of *P. multocida* from porcine bronchopneumonia was found. Compared to the human consumption of pork meat and *P. multocida* infections associated to humans, the presence of bronchopneumonia should be considered to constitute a minimal risk to the consumers as to infections with *P. multocida*.

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Table 1

Cultures of *P. multocida* and stage of gross lung lesions

Purity and semi-quantitative scoring of cultures of *P. multocida* isolated from lung lesions, pericardial sac and kidney of pigs with bronchopneumonia. Stage of gross lesions in the lungs of 114 cases of porcine bronchopneumonia cultivating positive for *P. multocida*.

Bronchopneumonia with isolation of <i>P. multocida</i>		Pure culture of <i>P. multocida</i>	Growth of <i>P. multocida</i> on blood agar		
			<i>Weak</i>	<i>Dense</i>	<i>Massive</i>
Lung (total)	114	97	17	21	76
Acute	9	8	0	2	7
Subacute ^a	22	17	3	9	10
Chronic ^b	83	72	14	10	59
Pericardial sac ^c	40 ^d	11	26	9	5
Kidney ^c	7 ^d	0	4	3	0

^aOne case of necrotizing pneumonia.

^bEight cases of necrotizing pneumonia.

^cFrom pigs with positive cultivation of *P. multocida* from bronchopneumonia (n=114)

^dFive pigs had positive culture of *P. multocida* from both kidney and pericardial sac; 35 pigs had isolation only from the pericardial sac and two pigs only from the kidney.

Table 2

Histopathology

Histological lesions in lung, heart and kidney from 114 cases of porcine bronchopneumonia cultivating positive for *P. multocida*. As control group, twenty pigs with no lung lesions and no cultures of *P. multocida* from lung, kidney or pericardial sac were included.

	No. of cases	Histopathology										
		Isolation of <i>P. multocida</i>			Lung: Bronchopneumonia						Heart	Kidney
		Lung	Pericardial sac	Kidney	Acute	Subacute	Chronic				Fibrotic pericarditis	Interstitial nephritis
							Necrotizing	Suppurative	Mixed	Nonsuppurative		
Bronchopneumonia	35	x	x		5	5	3	10	6	6	3	30 ^c
	2	x	-	x	-	1	1	-	-	-	(1 ^b)	2
	5	x	x	x	-	1 ^a	1	1	-	2	1	5
No lung lesions	72	x	-	-	4	15	3	22	17	11	14	51 ^d
	20	-	-	-	-	-	-	-	-	-	3	18
Total	134				9	22	8	33	23	19	21	106

^anecrotizing bronchopneumonia

^bone case of lymphocytic myocarditis

^cout of 34 kidneys examined

^dout of 68 kidneys examined

Table 3

In situ hybridization

Identification of *P. multocida* fluorescent *in situ* hybridization in selected cases of porcine bronchopneumonia cultivating positive for *P. multocida*. Selection of cases was based on the positive culture of *P. multocida* from the heart and/or kidney. As control groups, five animals only culturing *P. multocida* from bronchopneumonic lesions and five animals with no lesions and no culture of *P. multocida* were included.

Lesion in lung	Finding of <i>P. multocida</i> by cultivation/ <i>in situ</i> hybridization		
	<i>Lung</i>	<i>Pericardial sac</i>	<i>Kidney</i>
	35/30	35/-	-/2 ^a
Bronchopneumonia	2/2	-/-	2/1
(n=47)	5/5	5/-	5/1
	5/4	-/-	-/1 ^a
None (n=5)	-/-	-/-	-/-

^aFound in areas without lesions

Fig. 1

Sections from pigs with cranioventrally located bronchopneumonia and isolation of *Pasteurella multocida*.

A: Lung section with necrotizing pneumonia. Central core of necrosis (Ne) surrounded by neutrophils (N) and a rim of fibrosis (F). Lung. HE. Bar=50 µm.

B: Lung with suppurative bronchopneumonia. Marked infiltration dominated by neutrophils. Lung. HE. Bar=50 µm

C: Heart with fibrotic tissue (F) on the epicardium on the myocardium (M). HE. Bar = 50 µm.

D: Kidney with lympho-nodular hyperplasia. HE. Bar=50 µm.

E: Lung section with necrotizing pneumonia. *P. multocida* (red) are dispersed between inflammatory cells. Specific fluorescent *in situ* hybridization (FISH). Bar=10 µm.

F: Lung with suppurative bronchopneumonia. *P. multocida* (red) present within a bronchiole. FISH. Bar=20 µm