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Summary — Porcine tracheae maintained in culture were used in order to study the colonization by Mycoplasma hyopneumoniae. Rings excised from tracheae of newborn piglets were infected with M hyopneumoniae strain BQ 14 and, after different incubation times, were examined by light and electron microscopy. Non-infected tracheal mucosae maintained a normal appearance for several days. Infected tracheal rings showed progressive colonization with concomitant progressive damage to the mucosal surface. Early on during the infection, few mycoplasmas occurred over a ciliated epithelium. As the infection progressed, there was gradual loss of cilia; mycoplasmas tended to form microcolonies and to accumulate over the remaining ciliated cells. Mycoplasmas, first seen at the apex of the cilia, were then seen deeper in the interciliary space; some were even seen in contact with microvilli. In histological investigation, the final stage of the infection was characterized by a marked destruction of the epithelium with exfoliation of the epithelial cells. Infected mucosae showed typical damage caused by M hyopneumoniae, namely reduction of ciliary activity after 5 days, loss of cilia, and sloughing of ciliated cells. Our data indicate that porcine tracheal organ culture can be advantageously used to study colonization by M hyopneumoniae.

Mycoplasma hyopneumoniae / colonization / pig / trachea / organ culture

Résumé — Colonisation In vitro de la trachée porcine par Mycoplasma hyopneumoniae. Nous avons utilisé la trachée de porcelet maintenue en culture afin d’étudier la colonisation par Mycoplasma hyopneumoniae. Des anneaux de trachée ont été infectés avec la souche BQ 14, puis examinés par microscopie photonique et électronique après différents temps d’incubation. La muqueuse des anneaux non infectés a conservé un aspect normal durant plusieurs jours. Les anneaux infectés ont montré une colonisation progressive accompagnée d’un endommagement progressif de la muqueuse. Tôt après l’infection, quelques mycoplasmes ont été observés sur la surface ciliée de la muqueuse. Par la suite, nous avons observé une perte graduelle des cils; les mycoplasmes formaient des microcolonies et étaient retrouvés associés aux cils restants. Les mycoplasmes, premièrement observés à l’extrémité des cils, ont été retrouvés dans l’espace interciliaire, certains étaient même en contact avec les microvillosités. Histologiquement, le stade final de l’infection se caractérisait par une destruction marquée de l’épithélium. La muqueuse infectée a montré des dommages typiques de M hyopneumoniae, comme par exemple une réduction de l’activité ciliaire, la perte de cils et de cellules épithéliales. Nos résultats indiquent que la trachée de porcelet maintenue en culture peut être avantageusement utilisée pour étudier la colonisation par M hyopneumoniae.

Mycoplasma hyopneumoniae / colonisation / porc / trachée / culture d’organe

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INTRODUCTION

Mycoplasma hyopneumoniae is the causative agent of a worldwide chronic pneumonia in pigs known as swine enzootic pneumonia (Ross, 1986). The organism apparently causes ciliostasis and loss of cilia in the respiratory tract, but the mechanism of its pathogenicity is largely unknown (Debey and Ross, 1990).

Adherence of microbial pathogens to the mucosal surface of target tissues is an essential step in colonization of such surfaces (Ofek and Beachey, 1980). Adherence has been shown to be an important initial step in the pathogenesis of several mycoplasmal diseases (Razin, 1978). Adherence of M. hyopneumoniae to ciliated cells of the respiratory tract appears to be an important event in the development of enzootic pneumonia (Mebus and Underdahl, 1977; Tajima and Yagihashi, 1982). In a search for useful in vitro models of M. hyopneumoniae adherence, attachment of the organism to turkey red blood cells, monolayers of porcine kidney cells, and to human and porcine lung fibroblasts has been evaluated (Williams and Gallagher, 1978; Geary and Walzak, 1983; Young et al, 1989; Zielinski et al, 1990). Clear evidence that these in vitro models are good correlates of the adherence of M. hyopneumoniae to respiratory epithelia in infected pigs has not been obtained (Ross, 1990).

Tracheal organ cultures permit the study of the pathogenesis of organisms which damage respiratory mucosal cells by attachment and toxin production (McGee and Woods, 1978). These systems are easy to control and manipulate, and accurately mimic the natural host tissue configuration (Gabridge, 1984). Such in vitro cultures of differentiated tissue provide new and unique insight into the induction of cytotoxicity by mycoplasmas (Gabridge, 1984). Numerous human respiratory pathogens such as Neisseria meningitidis and Haemophilus influenzae (Stephens and Farley, 1991), Mycoplasma pneumoniae (Gabridge, 1984), and Bordetella pertussis (Bakaletz and Rheins, 1985) have been studied using tracheal organ cultures. Growth of Mycoplasma bovis has been investigated using bovine foetal trachea maintained in culture (Thomas et al, 1987). We have recently used porcine tracheal organ culture to study the colonization of Bordetella bronchiseptica (Dugal et al, 1990) and Actinobacillus pleuropneumoniae (Bélanger et al, 1990).

To the best of our knowledge, no studies have extensively examined the potential use of porcine tracheal organ culture to study the M. hyopneumoniae colonization process. The purpose of this study was to evaluate the usefulness of the aforementioned model in order to study M. hyopneumoniae colonization process and the damage this microorganism causes to the upper respiratory tract epithelium.

MATERIALS AND METHODS

Mycoplasma strain and growth conditions

M. hyopneumoniae strain BQ14, isolated from an outbreak of enzootic pneumonia in France, was identified by the disk growth inhibition test (Blanchard et al, 1992). Its identification was confirmed by EA Freunt, FAO/WHO collaborating center for Animal Mycoplasma, Aarhus, Denmark. M. hyopneumoniae was grown in Friis medium (Friis, 1975).

Tracheal rings method

Tracheal organ cultures were prepared according to Collier (1976) as modified by Dugal et al (1990) for porcine tracheae. Tracheae were excised from 10-day-old gnotobiotic piglets, then divided into rings between the cartilage with a sterile scalpel blade. The rings were placed in
24-well microplates, and incubated overnight at 37 °C with 0.4 ml of Eagle's minimal essential medium with Earle's salts (MEM) + 20 mM HEPES + bacitracin (200 µg/ml).

**Evaluation of ciliary activity**

At each step of the experiment, the rings were examined for ciliary activity by observation through an inverted microscope. Ciliary vigor was expressed as normal (+++), reduced (++), very reduced (+), or absent (-). Before infection, only rings showing normal vigor, ie those which exhibited a ciliary activity over more than 80% of the epithelial border, were used for the experiment.

**Infection**

Rings were rinsed 2–3 times with fresh MEM + 20 mM HEPES without bacitracin, and infected with 0.1 ml of the mycoplasmal culture containing 10^9 viable cells/ml. After a given period of time (ranging from 6 h to 9 days), ciliary activity was evaluated and some rings were prepared for histopathology and electron microscopy.

**Histopathology**

Tracheal rings were first fixed in buffered formalin, embedded in paraffin, and sectioned at 6 µm. Sections were stained with hematoxylin–phloxin–saffron (HPS) and Gram-stained.

**Scanning electron microscopy**

Tracheal rings were prepared for scanning electron microscopy as previously described (Bélanger et al, 1990; Dugal et al, 1990). Samples were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) and maintained at 4 °C overnight. They were dehydrated through graded ethanol, from 30–100%, critical point-dried in CO₂, mounted on aluminium stubs, and sputter coated with gold–palladium. They were then examined under a Hitachi S-530 microscope at an accelerating voltage of 20 kV.

**Transmission electron microscopy**

Tracheal rings were prepared for transmission electron microscopy as previously described (Dugal et al, 1990). Briefly, rings fixed as mentioned above were post-fixed with 2% osmium tetroxide for 2 h and dehydrated in a graded series of acetone washes. All the solutions used in processing the specimen, from post-fixation to dehydration with 70% acetone solution, contained 0.05% (wt/vol) ruthenium red. Samples were then washed twice in propylene oxide and embedded in Spurr low viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined under an electron microscope (Philips 201) at an accelerating voltage of 60 kV.

**RESULTS**

Porcine tracheal rings were used to study colonization of *M. hyopneumoniae* strain BQ14. The ciliary activity of non-infected and infected rings was first compared. Control non-infected rings retained their normal ciliary activity (+++) for more than a week after being in culture. Rings infected with *M. hyopneumoniae* showed a normal or reduced ciliary activity (+ to +++) for the first 5 days but afterwards progressively lost their activity and were completely inactive after 9 days. At various incubation times, some rings were formalin-fixed and prepared for histopathology. Control non-infected rings showed a normal mucosa covered by a ciliated epithelium for up to 9 days. Rings which were in contact with *M. hyopneumoniae* for 6 h had the same appearance (fig 1A). After a 24–30 h incubation with *M. hyopneumoniae*, the mucosa showed slight necrosis, with mycoplasmas seen as a dark lining at the apex of ciliated cells (fig 1B). After 48 h of incubation, a marked increase in epithelial necrosis and desquamation was observed (fig 1C). After 5 or more days of contact with mycoplasmas, the epithelium was completely ulcerated, and bacterial cells were seen associated with the remaining cilia (fig 1D).
Fig 1. Light micrographs of semi-thin sections of porcine tracheal rings stained with hematoxylin-phloxin-saffron. Shown are tracheal rings infected with \textit{M hyopneumoniae} strain BQ14 and incubated for 6 h (A), 30 h (B), 48 h (C), or 5 days (D). Bars = 10 μm.
Other rings were fixed and prepared for scanning electron microscopy. A control 
ring, in culture for 48 h, showed a normal 
mucosa almost entirely covered with ciliat-
ed cells (fig 2A). Rings infected with 
*M. hyopneumoniae* for 6 h looked the 
same, and some isolated mycoplasmas 
were occasionally seen at the tip of the cili-
a (fig 2B). After 24 h of incubation with 
*M. hyopneumoniae* the mucosal surface 
was still heavily ciliated, and numerous 
mycoplasmas forming microcolonies at the 
tip of the cilia were observed (fig 2C). After 
48 h of infection, tracheal rings were less 
ciliated than non-infected rings, and many 
mycoplasmas were associated with the cilia, 
either at their tip or occasionally deeper 
(fig 2D). A 5-day infected ring had a differ-
ent appearance, with cells sloughing from 
the surface; mycoplasmal microcolonies 
were associated with the remaining ciliated 
cells and some mycoplasmas were seen in 
contact with microvilli (fig 2E).

Transmission electron microscopy was 
also performed on non-infected and infect-
ed tracheal samples. Control tracheal rings 
were heavily ciliated (fig 3A) as observed 
previously by light and scanning electron microscopy. Six h after infection with 
*M. hyopneumoniae* BQ14, isolated myco-
plasmas were seen at the apex of the cilia. 
After 24 h mycoplasmas were observed 
forming microcolonies in close contact with 
cilia (fig 3B). Some ruthenium red-stained 
filaments were seen radiating from the 
bacterial cells.

**DISCUSSION**

An organ culture model, the tracheal rings, 
was used in order to study the colonization 
process of *M. hyopneumoniae* strain BQ14 
and to evaluate its effect on the epithelium of 
the porcine upper respiratory tract. This system appeared to be an adequate model 
for such a study.

Inverted microscopy showed that good 
ciliary activity could be preserved in control 
non-infected rings for at least 9 days, while 
infected rings retained their activity for the 
first few days but lost it completely after 9 
days. These results are in agreement with 
those of Debey and Ross (1990) who used 
an identical model. Williams and Gallagher 
(1978) did not observe ciliostasis or loss of 
ciliated cells before 5–6 days of infection 
using a similar model, but in which fibro-
blasts were also present.

Histopathological observations showed 
marked differences in the appearance of the 
epithelium fixed at different times after 
infection with this strain of *M. hyopneumon-
iae*. These observations are in full agree-
ment with the in vivo studies of Blanchard 

Electron microscopy confirmed the as-
sociation of the mycoplasmas with ciliated 
cells, more particularly to the cilia, and the 
formation of microcolonies. Results 
showed that early in the infection process 
*M. hyopneumoniae* was seen forming mi-
icrocolonies at the tip of the cilia, but later 
on mycoplasmas were seen deeper in the 
terciliary space and sometimes in contact 
with the microvilli. Since the present work 
was not a cinetic study we cannot con-
clude on the position shift of the mycoplas-
mas on ciliated cells as a function of time 
post-infection. However, according to this 
in vitro study, it is tempting to speculate 
that receptors for *M. hyopneumoniae* are 
not located exclusively at the apex of the 
cilia. Experimental infections in pigs with 
strain BQ14 did not show evidence of such 
a phenomenon (Blanchard et al, 1992). 
This is one of the first reports of a *M. hyop-
neumoniae* affinity for microvilli; such a 
type of contact has been reported by Taji-
ma et al (1985) following experimental in-
fications. We did not observe mycoplasmas 
in direct contact with the plasma mem-
brane, which confirmed previous results 
obtained in vivo (Mebus and Underdahl,
Fig 2. Scanning electron micrographs of critical-point dried preparations of porcine tracheal rings. Shown are a control, uninfected tracheal ring after 48 h in culture (A); tracheal rings infected with *M. hyopneumoniae* BQ14 and incubated for 6 h (B), 24 h (C), 48 h (D), or 5 days (E). Note in (B) an isolated bacterial cell seen at the tip of the cilia (arrow).
Results obtained in this in vitro study and in a previous in vivo experiment (Blanchard et al., 1992) suggest that the earliest response is the attachment of mycoplasma to the ciliated cells followed by a cytopathic effect and an exfoliation of epithelial cells. Damage to ciliary function is a characteristic of pathogenic bacteria that infect mucosal surfaces (Stephens and Farley, 1991). The diminished motility of the mucous blanket would provide a stable surface for attachment of these organisms and assist them in localizing within injured areas.

We did not observe a capsule layer covering the cells of *M. hyopneumoniae* BQ14 as was reported by Tajima and Yagihashi (1982). However, filaments stainable by ruthenium red were occasionally seen radiating from the cells. These filaments may represent capsular material which collapsed during preparation for electron microscopy. Damage typically associated...
with *M. hyopneumoniae* infection was reproduced in this model of organ culture. These data illustrate the utility of *in vitro* models of respiratory tissue for the study of mycoplasma infections, although these models could not completely reproduce the *in vivo* situation where immunity plays a role. Our study indicates that porcine tracheal organ culture is an important and relevant model to use in defining events such as attachment to ciliated and non-ciliated epithelial cells, ciliostasis, cytotoxicity, and multiplication which lead to successful colonization of the porcine respiratory tract.

**ACKNOWLEDGMENTS**

We thank P Morvan for invaluable technical assistance and CM Dozois for reviewing the manuscript. MJ received financial support from the National Research Council of Canada and the Ministère Français des Affaires Étrangères (Programme de Coopération Scientifique et Technologique Canada/France).

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