Tissue microarray and immunohistochemistry as tools for evaluation of antibodies against *Chlamydia*-like bacteria

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Tissue microarray technology was used to establish immunohistochemistry protocols and to determine the specificity of new antisera against various *Chlamydia*-like bacteria for future use on formalin-fixed and paraffin-embedded tissues. The antisera exhibited strong reactivity against autologous antigen and closely related heterologous antigen, but no cross-reactivity with distantly related species.

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INTRODUCTION

Several *Chlamydia*-like bacteria have recently been identified as potential emerging public health threats or pathogenic agents in animals. *Parachlamydia acanthamoebae*, '*Protochlamydia naegleriophila*' and *Simkania negevensis* have been reported as possible aetiological agents of pneumonia in humans (Casson *et al.*, 2008a; Corsaro & Greub, 2006; Friedman *et al.*, 2006; Greub *et al.*, 2003). *Parachlamydia* and related *Chlamydia*-like organisms have recently been reported in bovine abortion (Borel *et al.*, 2007). *Waddlia chondrophila* has also been implicated as an abortigenic agent in bovines (Henning *et al.*, 2002; Rurangirwa *et al.*, 1999), and a zoonotic potential for *W. chondrophila* was suggested by an association of anti-*Waddlia* antibodies in women and sustained contact with animals (Baud *et al.*, 2007). Infection may also occur following exposure to water colonized with infected free-living amoebae. Thus *Parachlamydia acanthamoebae* strain BN9 has been identified as a symbiont in free-living amoebae (Amann *et al.*, 1997), whereas *Parachlamydia acanthamoebae* strain Hall's coccus was identified within an amoeba isolated from a humidifier during investigation of an outbreak. '*Candidatus Protochlamydia amoebophila*' UWE25 was originally found in an *Acanthamoeba* isolate from a soil sample (Collingro *et al.*, 2005). '*Cribrlamydia sequanensis*' was isolated from Seine river water using amoebal co-culture (Thomas *et al.*, 2006) and *Neochlamydia hartmannellae* was detected in *Hartmannella vermiformis* isolated from the water conduit system of a dental care unit (Horn *et al.*, 2000).

To define further the possible pathogenic potential of these *Chlamydia*-like bacteria, new diagnostic tools are needed to demonstrate the agent within tissue lesions. Here, tissue microarray (TMA) technology associated with immunohistochemistry (IHC) was used to determine the specificity of new antisera against various *Chlamydia*-like bacteria and to investigate the level of cross-reactivity between different *Chlamydiales* species in formalin-fixed and paraffin-embedded pellets.

METHODS

Acanthamoeba castellanii (ATCC 30010) cultures were infected with *Parachlamydia acanthamoebae* strain Hall's coccus or strain BN9 (ATCC VR-1476), '*Candidatus Protochlamydia amoebophila*' strain UWE25 (ATCC PRA-7), '*Protochlamydia naegleriophila*' strain Knic, '*Cribrlamydia sequanensis*' strain CRIB-18, *S. negevensis* (ATCC VR-1471) and *W. chondrophila* (ATCC VR-1470). *H. vermiformis* strain BL was infected with *N. hartmannellae* (ATCC 50802). Uninfected *A. castellanii* and *H. vermiformis* cultures were prepared as negative controls.

Vero 76 (ATCC CRL-1587), Caco-2 (ATCC HTB-37) and HEp-2 (ATCC CCL-23) cells were infected with different human and animal chlamydial strains: *Chlamydia pneumoniae* strain Kajaaini 6, *Chlamydia abortus* strain S26/3 (sheep abortion), *Chlamydia psittaci* strain T49/90 (psittacosis agent), *Chlamydia pecorum* strain 1710S (swine origin), *Chlamydia trachomatis* strain LGV 434 and *Chlamydia suis* strain S45/6 (swine isolate). Uninfected cell cultures of each cell line served as negative controls. Amoebal and cell pellets were fixed in formalin and embedded in paraffin as described elsewhere (Borel *et al.*, 2006).

A cell culture array block including two equally prepared sets of cell and amoebal pellets was created with the TMA Builder (Histopathology; <http://www.histopat.hu>) according to the manufacturer's instructions. Briefly, the recipient paraffin block with 24 holes

Abbreviations: IHC, immunohistochemistry; TMA, tissue microarray.

of 2 mm diameter each arranged in four columns and six rows was built using the TMA Builder. The whole cell pellets from the donor blocks were punched out with a Paraffin-Punch-Extractor and arrayed in a preformed recipient paraffin block according to the protocol. Four-micrometre sections were cut using a standard microtome.

For IHC, two commercially available chlamydial antibodies were used: a *Chlamydiaceae* family-specific mouse monoclonal antibody (mAb) directed against the chlamydial LPS (mLPS; clone ACI-P; Progen) and a *Chlamydia*-specific mouse mAb (IgG1) directed against recombinant *Chlamydia trachomatis* Hsp60 (clone A57-B9; Milan Analytica).

Polyclonal mouse antisera for the detection of various *Chlamydia*-like bacteria were produced previously and characterized by immunofluorescence and Western blotting (Casson *et al.*, 2007). Optimization

experiments for antigen retrieval and appropriate dilution of antisera for IHC were performed using infected amoebal and cell pellets. Primary antisera were then applied to the TMA as follows: (i) mLPS at a dilution of 1:50, (ii) anti-Hsp60 at a dilution of 1:1200, (iii) antiserum against *Parachlamydia acanthamoebae* strain Hall's coccus at a dilution of 1:6000, (iv) antiserum against '*Protochlamydia naegleriophila*' strain Knic at a dilution of 1:20 000 and (v) antisera against *Parachlamydia acanthamoebae* strain BN9, '*Criblamydia sequanensis*' and *W. chondrophila* at dilutions of 1:40 000.

Detection was performed with a detection kit (ChemMate; Dako) according to the manufacturer's instructions. Briefly, paraffin sections were deparaffinized in xylene and rehydrated through graded ethanol to water. Antigen retrieval was performed by 5 min enzyme digestion (Pronase; Dako) for the mLPS antibody, and repeated microwave heating (750 W for 10 min) in citrate buffer, pH 6.0 (Target Retrieval

Fig. 1. (a) Uninfected *A. castellanii*. IHC with mouse antiserum against *Parachlamydia acanthamoebae* strain Hall's coccus (1:6000). (b, c) *A. castellanii* infected with *Parachlamydia acanthamoebae* strain Hall's coccus. IHC with mouse antiserum against *Parachlamydia acanthamoebae* strain Hall's coccus (1:6000). (d) *A. castellanii* infected with '*Criblamydia sequanensis*'. IHC with mouse antiserum against '*Criblamydia sequanensis*' (1:40 000). (e) *A. castellanii* infected with '*Protochlamydia naegleriophila*' strain Knic. IHC with mouse antiserum against '*Protochlamydia naegleriophila*' strain Knic (1:20 000). (f) *A. castellanii* infected with '*Criblamydia sequanensis*'. IHC with mouse antiserum against *W. chondrophila* (1:40 000). (g) HEp-2 cells infected with *Chlamydophila pneumoniae*. IHC with the chlamydial mLPS (1:50). (h) HEp-2 cells infected with *Chlamydophila pneumoniae*. IHC with a chlamydial anti-Hsp60 antibody (1:1200). (i) Uninfected *A. castellanii*. IHC with a chlamydial anti-Hsp60 antibody (1:1200). In all sections, the secondary antibody was detected using 3-amino-9-ethylcarbazole/peroxidase and counterstained with haematoxylin. Magnifications: $\times 400$ (a, b, d–f); $\times 1000$ (c); $\times 200$ (g–i).

Solution, ChemMate; Dako), for the antisera against *Chlamydia*-like bacteria. Primary antibodies were incubated for 1 h. For inhibition of endogenous peroxidase activity, the slides were immersed in peroxidase-blocking solution (Dako) for 5 min at room temperature. Negative and positive controls for each section were included as described elsewhere (Borel *et al.*, 2006).

RESULTS AND DISCUSSION

The mLPS and anti-Hsp60 antibodies, as well as the antisera against the different *Chlamydia*-like bacteria, did not exhibit cross-reactivity against uninfected cell or amoebal pellets (Fig. 1a, i). Table 1 shows the antibody titres and cross-reactivities of the different chlamydial antibodies with *Chlamydiaceae* and *Chlamydia*-like bacteria, as determined by IHC on the TMA. In general, the antisera exhibited strong reactivity to autologous antigens in amoebal pellets by IHC (Fig. 1b–e). Significant cross-reactivity was observed between closely related species, for instance between *Parachlamydia* and ‘*Protochlamydia*’ strains. In contrast, no or little cross-reactivity was detected between distantly related *Chlamydia*-like bacteria. Surprisingly, the anti-*Waddlia* antibody cross-reacted with the ‘*Criblamydia sequanensis*’-infected amoebal pellet (Fig. 1f) but not vice versa. Cross-reactivity of the antiserum against *Parachlamydia acanthamoebae* strain Hall’s coccus with ‘*Protochlamydia naegleriophila*’ and *N. hartmannellae* was observed once, but could not be confirmed in another experiment and was therefore interpreted as questionable. Vero 76, Caco-2 and HEp-2 cells infected with *Chlamydiaceae* strains remained negative when tested with all polyclonal mouse antisera, indicating no cross-reactivity between any *Chlamydiaceae* species and the *Chlamydia*-like bacteria investigated here. The mLPS

antibody reacted with the *Chlamydiaceae* strains as expected (Fig. 1g), but not with *Chlamydia*-like bacteria. The same was observed for the anti-Hsp60 antibody (Fig. 1h), except for some cross-reactivity to the pellet infected with *N. hartmannellae*.

Antisera against *Parachlamydia acanthamoebae* strain Hall’s coccus and *W. chondrophila* have already been applied successfully to formalin-fixed and paraffin-embedded bovine placenta specimens and resulted in the first report of *Parachlamydia* in bovine abortion (Borel *et al.*, 2007). The antibody against *Parachlamydia acanthamoebae* strain Hall’s coccus has also been successfully applied to lung samples from mice experimentally infected with *Parachlamydia acanthamoebae*: IHC results of lungs correlated well with histopathological lesions and real-time PCR (Casson *et al.*, 2008b).

Reactivity to LPS was not observed in any *Chlamydia*-like bacteria tested. This suggests that these species do not have a LPS or possess a truncated LPS, as shown for ‘*Candidatus Protochlamydia amoebophila*’ UWE25 (Horn *et al.*, 2004). Thus these new agents will not be detected by routine diagnostics when using an antibody directed against chlamydial LPS. Knowledge of the presence of heat-shock proteins in *Chlamydia*-like bacteria is scarce. Nevertheless, some cross-reactivity of the anti-Hsp60 antibody could be observed in the *N. hartmannellae*-infected amoebal pellet, but not in *H. vermiformis* pellets, suggesting the presence of a heat-shock protein-like structure in this species.

In conclusion, TMA technology in combination with IHC is a useful tool for testing the specificity of antibodies for their future use on formalin-fixed and paraffin-embedded tissues. Possible cross-reactivity of antibodies in closely

Table 1. Dilutions and cross-reactivities of different antibodies with *Chlamydiaceae* and *Chlamydia*-like bacteria, as determined by IHC on TMA

Species studied	<i>Parachlamydia acanthamoebae</i> strain Hall’s coccus	<i>Parachlamydia acanthamoebae</i> strain BN9	‘ <i>Protochlamydia naegleriophila</i> ’ strain Knic	‘ <i>Criblamydia sequanensis</i> ’	<i>Waddlia chondrophila</i>	LPS	Hsp60
<i>Parachlamydia acanthamoebae</i> strain Hall’s coccus	1:6000	1:40 000	0	0	0	0	0
<i>Parachlamydia acanthamoebae</i> strain BN9	1:6000	1:40 000	0	0	0	0	0
‘ <i>Protochlamydia naegleriophila</i> ’ strain Knic	0	0	1:20 000	0	0	0	0
‘ <i>Candidatus Protochlamydia amoebophila</i> ’ strain UWE25	1:6000*	0	1:20 000	0	0	0	0
‘ <i>Criblamydia sequanensis</i> ’	0	0	0	1:40 000	1:40 000	0	0
<i>Simkania negevensis</i>	0	0	0	0	0	0	0
<i>Waddlia chondrophila</i>	0	0	0	0	1:40 000	0	0
<i>Neochlamydia hartmannellae</i>	1:6000*	0	0	0	0	0	1:1200
<i>Chlamydiaceae</i>	0	0	0	0	0	1:50	1:1200

*These results were interpreted as questionable, as they were not confirmed in another experiment.

related species should be considered when investigating human or animal tissues with these antisera by IHC.

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