Characterisation of *Pasteurella multocida* isolated from rabbits in the Czech Republic

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ABSTRACT: Twenty seven *Pasteurella multocida* rabbit isolates were characterised by pulsed-field gel electrophoresis (PFGE) generated by restriction endonuclease *Apa*I, and examined for the presence of capsule and fimbriae, and tested of haemolytic activity and endotoxin release in presence of rabbit plasma. Among the isolates a high number (n = 20) of different PFGE types was observed. All isolates were found to be encapsulated and four of them also possessed fimbriae. Although the presence of the *aph*A gene, conferring a haemolytic phenotype for *Escherichia coli* under anaerobic conditions, was indicated in all of the isolates by PCR, clear haemolytic activity was observed in only one isolate when grown anaerobically. All of the isolates grew easily in the presence of rabbit plasma which showed a high capacity to bind the released endotoxin.

Keywords*:* pasteurellosis; PFGE; RFLP; LPS; haemolysis; virulence factors

The Gram-negative bacterium *Pasteurella multocida* exhibits a broad host range including most mammals, birds and also humans (Adlam and Rutter, 1989; Quinn et al., 1994). Pasteurellosis is one of the most significant bacterial diseases of rabbits and one of the major causes of considerable economic loss in large production units throughout the world (Manning, 1982; Takashima et al., 2001). The disease is characterised by various clinical syndromes, e.g. respiratory distress, genital affections, abscesses and septicemia, but infection by *P. multocida* can also appear without any clinical signs manifested (DiGiacomo et al., 1983; Delong and Manning, 1994). The variability in clinical signs as well as the course of the disease may be influenced by different *P. multocida* virulence factors such as a capsule, fimbriae, lipopolysaccharides (endotoxin), dermonecrotoxin, neuraminidase etc. (Maheswaran and Thies, 1979; Glorioso et al., 1982; Rhoades and Rimler, 1987; DiGiacomo et al., 1989; Straus et al., 1996). Capsular typing of *P. multocida* rabbit isolates has shown that to date, pasteurellosis in rabbits is mainly caused by the capsular type A and, to a lesser extent, capsular type D strains (Chengappa et al., 1982; Kawamoto et al., 1990; Vandyck et al., 1995; Dabo et al., 1999). However, the finding of the capsular type F in a rabbit host has been recently reported, too (Jaglic et al., 2004). A new generation of typing systems based on molecular techniques allows a more precise discrimination among rabbit isolates of *P. multocida* and improves the epizootiological study of the disease (Al Haddawi et al., 1999; Dabo et al., 1999, 2000; El Tayeb et al., 2004; Jaglic et al., 2005).

This study was aimed at a heterogeneous collection of *P. multocida* rabbit isolates, among which the clear domination of a single ribotype was found in our previous work (Jaglic et al., 2005). In an effort to achieve better discrimination among the isolates, and to find out if a single clone truly prevails in the rabbit population of the Czech Republic, the same isolates were additionally characterised by pulsed-field gel electrophoresis (PFGE). The incidence of selected virulence factors (i.e. capsule, fimbriae and haemolysins) and the influence of rabbit plasma on endotoxin release *in vitro* were also analysed in this study.

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MATERIAL AND METHODS

***P. multocida* isolates and strains, and growth conditions**. Twenty seven *P. multocida* rabbit isolates included in this study (Table 1) have already been characterised by capsular PCR typing and ribotyping, and also determined as dermonecrotoxin negative in our previous works (Jaglic et al., 2004, 2005). One of these isolates was designated as a reference strain J-4103 and is available at the Czech Collection of Animal Pathogenic Microorganisms (CAPM 6431). The isolates were obtained from different locations in the Czech Republic, and they were limited to one per rabbit nest except for those (two pairs of isolates) which were of different capsular types. For PFGE analysis, additional four *P. multocida* reference strains (14070/82, W-9217, 45/78, and 93 IOWA), originating from pigs and cattle, were also included in this study. *P. multocida* reference strain X-73, in which the presence of the *ahpA* gene was formerly confirmed by southern hybridisation (Cox et al., 2000), was also used in the testing of haemolytic activity. The isolates and strains were grown at 37°C on blood agar (Blood Agar Base No. 2, HiMedia) containing 5% of sheep blood.

PFGE. PFGE was carried out according to Gunawardana et al. (2000) with a few modifications. Briefly, an appropriate volume of the overnight brain hearth infusion (BHI) culture containing approximately 10^9 cells was used for the DNA preparation, and cell lysis was performed in the presence of RNase at a concentration of 0.01 mg/ml. DNA was digested with 25 U of *Apa*I at 25°C for 19 h in digestion buffer containing bovine serum albumin at a concentration of 100 µg/ml (New England BioLabs, Inc.). Electrophoresis in Tris-borate-EDTA (TBE) buffer was carried out on the CHEF-DR III System (Bio-Rad, USA) with a voltage of 6 V/cm for 22 h with an initial switch time of 1 s, increasing to 30 s.

Detection of capsules and fimbriae. Indirect detection of the capsules was performed by cultivation of the isolates in presence of capsule depolymerising mucopolysaccharidases (bovine testes hyaluronidase, heparinase III, and chondroitinase AC; all from Sigma Chemical) according to Rimler

Table 1. List of the *Pasteurella multocida* rabbit isolates examined in this study

No. of isolates	Site of isolation	Herd status	Year of isolation
1	data not available	data not available	1967
1	lungs	rhinitis, pneumonia	1997
2	blood	rhinitis, pneumonia	1997
1	data not available	data not available	1999
1	nose	data not available	2000
1	lungs	rhinitis, pneumonia	2000
1	nose	rhinitis, pneumonia	2000
1	uterus	acute septicemia	2001
6	lungs	data not available	2001
2	nose	rhinitis, pneumonia	2001
1	nose	data not available	2001
1	conjunctiva	data not available	2001
1	data not available	data not available	2001
1	data not available	data not available	2002
1	lungs	rhinitis, pneumonia	2003
1	lungs	rhinitis, pneumonia	2004
1	nose	rhinitis	2004
1	nose	data not available	2004
1	heart	data not available	2004
1	lungs	data not available	2004

(1994). Encapsulated isolates were subjected to decapsulation which was demonstrated by a zone of colonies reduced in size.

Direct demonstration of the capsule and fimbriae was performed by electronic microscopy (Philips 208 Morgagni, The Netherlands). Drops from 6 hours old bacterial cultures grown in LB-broth (Difco, USA) at 37°C were placed on Parafilm and covered by 200-mesh Formvar-carbon-coated copper grids (Agar Scientific, England) for 20 min at room temperature. Detection of the capsule was performed according to Huebner et al. (1999). Briefly, the grids were blocked by their placement on 5-µl drops of 0.5% fish-skin gelatine in phosphate-buffered saline containing 0.1% Tween 20 (PBS-Tween) for 10 min. To mark out the capsule, specific rabbit hyperimmune antisera were applied onto the grids at a 1:5 dilution (preimmune rabbit serum was used as a negative control). The hyperimmune sera were prepared by intra-muscular immunisation of New Zealand white rabbits with capsular type A (14070/82), D (45/78) and F (J-4103) *P. multocida* strains: one ml of the PBS suspension containing approximately 10^{10} bacterial cells inactivated by 0.5% formalin was applied three times in three-week intervals, and three weeks after the last application the rabbits were bled (an equal volume of complete (prior to the first application) or incomplete (prior to the following applications) Freund's adjuvant (Difco, USA) was added to the bacterial suspension). The grids with sera were incubated for 20 min at room temperature and washed once with 0.1 × PBS-Tween. Protein A-20 nm colloidal gold (Sigma, USA) was then applied onto the grids at a dilution of 1:10. After incubation for 20 min at room temperature, the grids were washed four times with deionized water. Bacterial cells were observed without staining at a magnification of 10 000×. For detection of the fimbriae the grids previously incubated with bacterial suspensions were stained by 1% phosphotungstic acid, and bacterial cells were observed at a magnification of 25 000×.

Haemolytic activity. The isolates and the reference strain X-73 were tested for the *aph*A gene by PCR and also cultivated on different blood agars.

For the PCR examination, two specific primers – AHPAF (5´-CTT TTA GCG CAC ACC AGT CA-3´) and AHPAR (5´-CCA ATA ATA CCC CAG CCA GA-3´) – were designed according to the published *ahp*A sequence (Cox et al., 2000). The expected size of the resulting amplicon was 247 bp. To release bacterial DNA, a single bacterial colony was resuspended in 100 µl of distilled water and boiled for 15 min. After brief centrifugation, 2 µl of supernatant containing the genomic DNA was used for PCR amplification. PCR was carried out using *Taq*PCR Master Mix Kit (Qiagen, Germany) in a final volume of 20 µl. For amplification the PCR mixtures were denatured by incubation at 94°C for 5 min followed by 30 cycles of (i) denaturation at 94°C for 30 s, (ii) primer annealing at 58°C for 30 s, and (iii) elongation at 72°C for 30 s. The samples were then incubated at 72°C for 5 min for completion of the elongation process of the final PCR products. The amplification products were separated by electrophoresis in 2% agarose gel in TBE buffer and visualised by ethidium bromide staining.

To assess haemolytic activity, the isolates were cultivated on different blood agars. The culture medium contained 4.5 g of VF base (93.3% peptone, 6.7% NaCl; Imuna Pharm, Slovakia), 1.6 g of agar base, 1 g of tryptone, 0.5 g of yeast extract, 0.5 g of dextrose, 0.5 g of K_3PO_4, 0.05 g of L-cysteine HCl, and 0.1 ml of 10 mg/ml vitamin K in 100 ml of distilled water. One hundred ml of the culture medium were supplemented by 10 ml of horse, sheep or rabbit whole blood, or by 10 ml of 40% suspension of horse, sheep or rabbit washed erythrocytes. Erythrocytes were washed for three-times in the appropriate volume of PBS and 32.4 mg of $CaCl_2$ were added to 100 ml of the culture medium. Incubation was carried out at 37°C for 48 h under both aerobic and anaerobic conditions. Anaerobic incubation was performed in anaerobic jars using AnaeroGen sachets (Oxoid, UK) generating an atmosphere of oxygen level below 1%. Two haemolytic reference strains, *Streptococcus pneumoniae* (6B) and *Fusobacterium necrophorum* (142D), were used as controls.

Endotoxin release. The isolates were prepared for endotoxin assay according to Lee et al. (1992) with a few modifications. In brief, cells from 200 µl of overnight BHI cultures were pelleted at 8 220 × g for 10 min and resuspended in 200 µl of fresh BHI and incubated at 37°C for 2 hours. Ten µl of each sample were inoculated into 1 ml of LB-broth and 1 ml of LB-broth containing 50% heparinised rabbit plasma obtained from specific pathogens-free rabbits (Charles River Laboratories, Germany GmbH), and then incubated at 37°C in a shaker. Absorbance of the cultures was measured at 605 nm (SmartSpec 3000, Bio-Rad, USA) after 4, 8, and 12 h of growth, and at the same intervals, aliquots were removed.

Cells were removed from the aliquots by centrifugation at 8220 × g for 10 min and the supernatants were stored at –70°C until assayed.

The supernatants from five isolates (including J-4103) showing different tendencies of growth were selected for endotoxin assay which was carried out by the Chromogenic Limulus Amebocyte Lysate Test (Cambrex, USA) according to the manufacture's instructions. The blank culture media (LB-broth and LB-broth supplemented by plasma) were also tested as negative controls. The data of absorbance values and endotoxin amounts were statistically analysed by paired *t*-test using the statistical program STAT Plus (Matouskova et al., 1992).

RESULTS

PFGE

PFGE was found to be a very accurate method in typing of *P. multocida* rabbit isolates revealing a high diversity among them. The restriction analysis of chromosomal DNA using *Apa*I resulted in twenty (I - XX) different profiles (PFGE-types) among 27 *P. multocida* rabbit isolates (Figure 1) what was much more than had been the number (n = 6) of previously identified ribotypes among the same isolates. Most of the PFGE-types (13 of 20) occurred in only one isolate, and the remaining PFGE-types were detected in two isolates. PFGE-types generally correlated with the capsular types and ribotypes of *P. multocida*. Only three PFGE-types (III, IX and XIII) were found in two different capsular types or ribotypes (Table 2).

Four distinct PFGE-types (XXI–XXIV), which were not observed among the rabbit isolates, were found in four reference strains originating from pigs (n = 3) and cattle (n = 1) (Figure 1).

Expression of capsule and fimbriae, and haemolytic activity

Although great variability among the isolates was detected by PFGE, most of the isolates were found to be uniform, in connection with the expression of monitored virulence factors. As it previously remarked, all isolates were formerly determined as dermonecrotoxin negative. All of the isolates also possessed the capsule that was confirmed by both methods used in this study. Fimbriae were detected in only four isolates belonging to the capsular types A (n = 3) and F (n = 1), all of them also of a different restriction (ribotype/PFGE-type) pattern 1/XVIII, 2/XIII, 4/XII, and 6/VI. The capsule and fimbriae are shown in Figure 2.

All of the *P. multocida* isolates, as well as the reference strain X-73, were positive by PCR for the *aph*A gene. However, only a single isolate belonging to the capsular type F (restriction pattern 1/XVI) produced an obvious haemolysis which surrounded the colonies and which was observed on the washed horse and sheep erythrocytes under anaerobic conditions. None of the remaining isolates (including the reference strain X-73) showed a clear haemo-

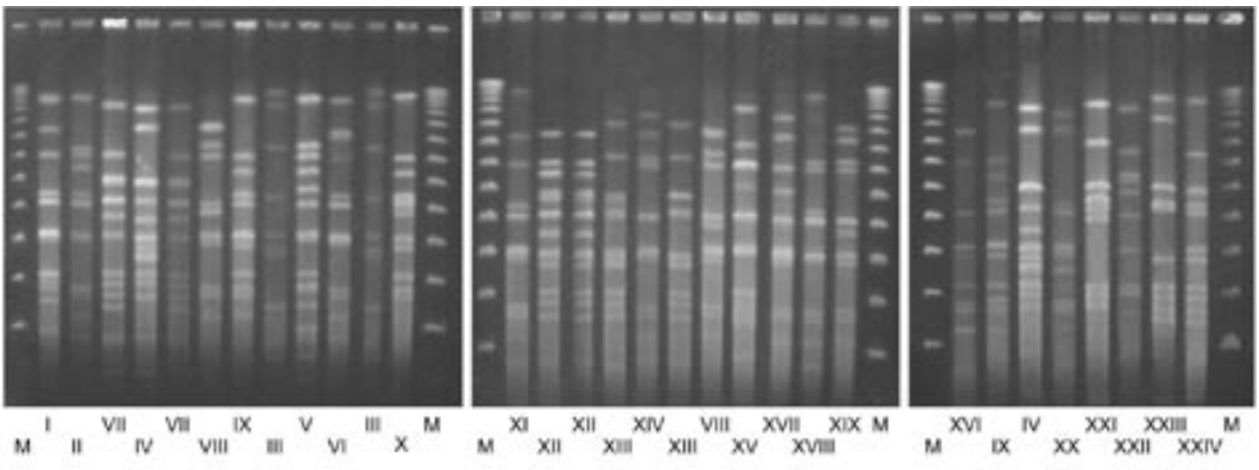

Figure 1. PFGE patterns of *Pasteurella multocida* isolates and reference strains after digestion with enzyme *Apa*I. PFGE-types I–XX were found among the rabbit isolates, PFGE types XXI–XXIV were found among the reference strains. M = 50 Kb Lambda DNA ladder of the range of 0.05 Mb to 1 Mb (Bio-Rad, USA)

Table 2. Correlation among capsular types, ribotypes and PFGE-types of *Pasteurella multocida* isolated from rabbits

No. of isolates	Capsular type	Ribotype[a]	PGFE Type
9	A	1	I, II, III, IX, XI, XV, XVIII, XIX, XX
5	A	4	IV, XII, XIII
1	A	5	V
2	A	6	III, VI
2	D	3	VII
7[b]	F	1	VIII, IX, X, XIV, XVI, XVII
1	F	2	XIII

[a]as defined by Jaglic et al. (2005)

[b]including the reference strain J-4103 which was of PGFE Type VIII

lytic activity on any of the blood agars used in this study. However, a very weak or doubtful haemolysis which was restricted only to sites of very plentiful and intensive growth, and, in general, visible only after removing the culture was observed in some isolates grown both aerobically and anaerobically. This very weak haemolytic activity was very rarely recorded on the agar plates containing the whole blood; however, it was found in most isolates growing on the agars prepared from the washed erythrocytes (mainly of horse origin and the least frequently of rabbit origin).

Endotoxin release

Damage or lysis of Gram-negative bacteria, caused by various endogenous factors such as complement and other bactericidal proteins, results in endotoxin release that could lead to endotoxic shock. The influence of rabbit plasma on endotoxin release and survival of the isolates in its presence were therefore analysed in this study. To our surprise a significant and intensive growth rate in the LB-broth containing the plasma was observed among the isolates when compared with their growth in the LB-broth alone where some cultures showed a decrease in their density after four or eight hours of incubation. However, the amounts of endotoxin found in the presence of plasma were much lower than those found in the LB-broth alone (Table 3). This could have been a consequence of a lower sensitivity of the isolates to the complement and other bactericidal proteins, but also of the neutralisation of free endotoxin by plasma components. The rabbit plasma was therefore tested on its ability to bind and

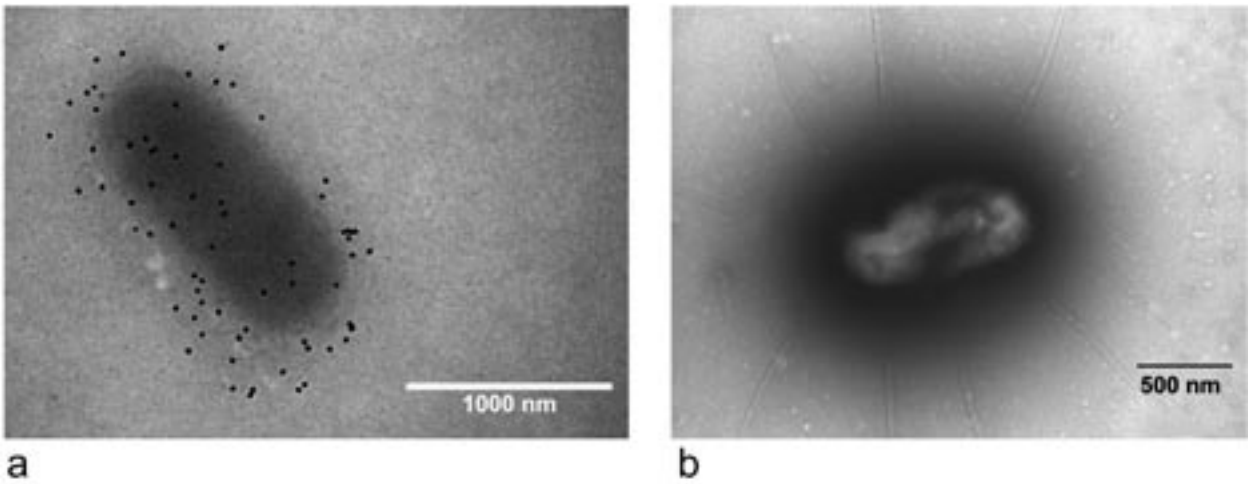

Figure 2. Electron microscopy of capsule (a) and fimbriae (b) found in *Pasteurella multocida* isolates. The capsule is indicated by gold particles (black spots) bound to Protein A

Table 3. Endotoxin amounts found among the five selected *Pasteurella multocida* isolates regarding the absorbance value, time of incubation, and culture medium

Isolate	LB-broth						LB-broth + plasma					
	4 hours		8 hours		12 hours		4 hours		8 hours		12 hours	
	A[1]	E[2]	A	E	A	E	A	E	A	E	A	E
15/02	0.57	5.2	0.37	8.5	0.31	11.5	1.24	1.8	1.96	2.4	2.18	3.0
J-4103	0.70	2.8	1.26	4.3	1.47	7.2	1.40	0.9	2.08	3.5	2.34	5.0
42/03	0.86	2.2	1.29	2.6	1.66	3.6	1.46	1.1	2.35	1.6	2.60	2.1
49/04	0.07	3.8	0.29	13.7	0.12	14.1	0.23	1.1	1.23	8.1	1.33	12.5
77/04	0.55	1.4	1.29	6.0	1.45	8.6	0.32	0.1	1.29	0.8	1.98	1.4
Mean	0.55	3.08[c]	0.90[a]	7.02[d]	1.00[b]	9.00[e]	0.93	1.00[c]	1.78[a]	3.28[d]	2.09[b]	4.80[e]
SD	0.30	1.47	0.52	4.32	0.73	4.03	0.60	0.61	0.50	2.87	0.48	4.51

[1]absorbance at 605 nm; [2]endotoxin units/ml × 10^4; [aa, dd, ee]$P < 0.05$; [bb, cc]$P < 0.01$

neutralise endotoxin. The supernatants obtained from 12 hour old non-supplemented LB-broth cultures were mixed with plasma and LB-broth alone in a 1:1 ratio and after incubation at 37°C for 2 h additionally assayed. The addition of plasma resulted in approximately 60–90% decrease in endotoxin levels (Figure 3) while the addition of LB-broth alone was not of significant influence on endotoxin concentrations (data not shown).

DISCUSSION

Due to their great discriminatory power, DNA-based typing methods have been already established as an effective tool in the characterisation of *P. multocida* (Blackall and Miflin, 2000). Among them, pulsed-field gel electrophoresis seems to be one of the most suitable techniques used in clinical microbiology (Tenover et al., 1995). During

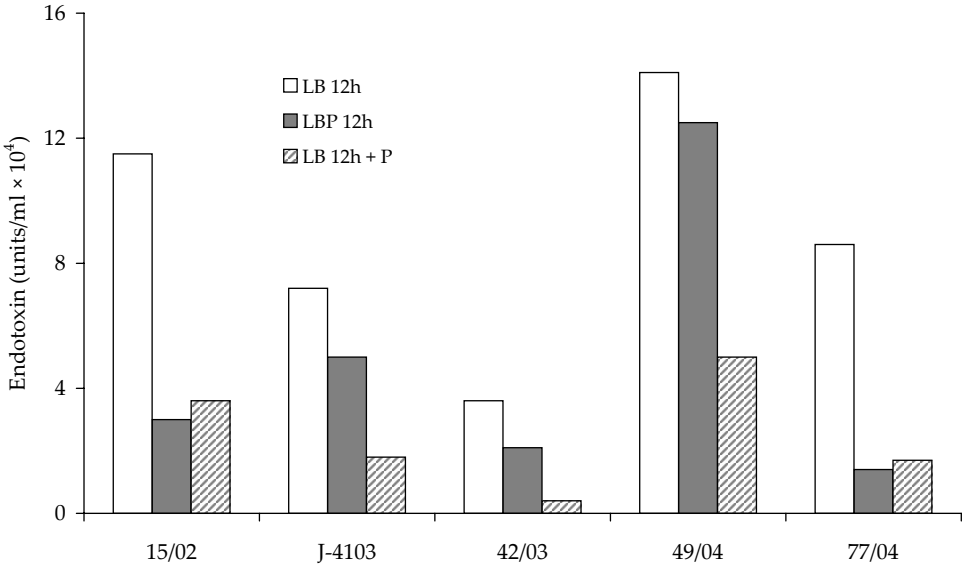

Figure 3. Endotoxin amounts found after 12 hours of growth in non-supplemented LB-broth (LB 12h), after 12 hours of growth in LB-broth supplemented by rabbit plasma (LBP 12h), and after exposure of the supernatants obtained from 12 hours old non-supplemented LB-broth cultures to rabbit plasma (LB 12h + P)

the past, PFGE prove to be a valuable technique in molecular epizootiology of *P. multocida* as reviewed by Hunt et al. (2000a). More recently, Kiss et al. (2004) also recommended PFGE as a useful tool in *P. multocida* typing. Up to now, PFGE has been mainly used in the typing of avian, porcine, and cattle *P. multocida* isolates (Donnio et al., 1994; Townsend et al., 1997; Gunawardana et al., 2000; Lainson et al., 2002; Pedersen et al., 2003; Kardos and Kiss, 2005), and, to our knowledge, it has not been used in characterisation of rabbit isolates, yet.

In the previous work (Jaglic et al., 2005) we found that only one ribotype dominated among the rabbit isolates in the Czech Republic (16 isolates were of the same ribotype 1), and as PFGE has been recommended as an accurate method in typing of *P. multocida*, we were interested in whether PFGE would confirm the previous classification generated by ribotyping or to allow a more accurate discrimination. Unlike Gunawardana et al. (2000) who characterised 22 turkey isolates by PFGE with discriminatory power similar to ribotyping (Blackall et al., 1995), we observed a significantly higher number of PFGE-types (n = 20) than ribotypes (n = 6) that indicates that almost all of the rabbit nests examined in this study were colonised by a different clone of *P. multocida*. Similar to our finding, Townsend et al. (1997) achieved a greater discrimination among haemorrhagic septicaemia-causing *P. multocida* isolates when they used PFGE instead of ribotyping.

The presence of the capsule mainly increases the resistance of a *P. multocida* cell to phagocytosis and enhances its virulence potency when compared with non-capsulated strains (Maheswaran and Thies, 1979; Borrathybay et al., 2003). The fact that *P. multocida* is a commonly encapsulated organism is supported by this study, too. On the other hand, the fimbriae which can play important role in colonisation of host tissue (Doughty et al., 2000; Al Haddawi et al., 2000) have not been described so frequently in *P. multocida* (Grund et al., 1990), and, in this study, they were found in only four isolates. However, a potential for their production, which did not have to be expressed under the conditions used in this study, can not be excluded among the remaining isolates.

Although *P. multocida* has been generally considered as a non-haemolytic bacterium some studies have reported on its haemolytic activity which could be another virulence factor expressed by this microorganism. Lysis of erythrocytes by *P. multocida* was already noticed by Lee et al. (1990, 1991) who in their later study observed that a virulent avian isolate, but not the low virulent CU vaccine strain, displayed haemolysis. A relatively strong haemolytic activity of anaerobically grown *P. multocida* was described by Cox et al. (2000) and Hunt et al. (2000b) who identified two *P. multocida* genes, *ahp*A and *mes*A, which conferred a haemolytic phenotype on *Escherichia coli* under anaerobic conditions. However, the authors suggested that the AhpA and MesA proteins are not anaerobically expressed haemolysins per se, but possibly affect regulation and the expression of silent cryptic haemolysin SheA under anaerobic conditions. In our study we indicated the presence of *ahp*A gene in all of the isolates examined but the obvious haemolysis was observed only in one isolate. On the other hand, Diallo and Frost (2000) described lysis of washed erythrocytes by *P. multocida* extract obtained from both aerobic and anaerobic cultures, the activity of which was eliminated by exposure to serum or serum albumen. The authors suggested that the factor(s) mediating haemolysis may be cell-bound. This might be in agreement with our findings of non-diffused weak haemolysis which was strictly restricted to the sites of intensive growth and which was mainly found on washed erythrocytes under both aerobic and anaerobic conditions.

Lipopolysaccharide-protein complex (endotoxin) is an important constituent of the outer membrane of a *P. multocida* cell. Besides its toxic role in pathogenesis of the disease (Schimmel et al., 1982; Mendes et al., 1994; Kunkle and Rimler, 1998; Horadagoda et al., 2002), endotoxin is also immunogenic at low concentrations (Rimler and Phillips, 1986; Muniandy et al., 1998). LPS molecules are not toxic when they are bound to the bacterial outer membrane but after release from the bacterial wall they are exposed to immune cells and evoke an inflammatory response (Van Amersfoort et al., 2003). LPS is released from the bacterial cells when they multiply but also when bacteria are damaged or lysed by various factors present in plasma such as complement and other bactericidal proteins (Rietschel et al., 1994; Smedsrod et al., 1994). Moreover, a simple saline extraction of *P. multocida* endotoxin (Ganfield et al., 1976) further indicates its loose bond to the cell surface. In an attempt to assess the influence of plasma on endotoxin release *in vitro*, we compared the amounts of endotoxin released from the rabbit isolates when they were grown in LB-broth alone and in LB-broth supple-

mented by rabbit plasma. Unlike our finding, Lee et al. (1992) reported higher amounts of endotoxin released from two avian strains when they were grown in the presence of turkey plasma instead of LB-broth alone. They explained it by a complement activity although the strain which was more complement resistant released more endotoxin than the other one. Intensive growth in the presence of plasma accompanied by relatively low amounts of endotoxin released indicated that the isolates examined in this study were either resistant to the complement and other bactericidal proteins, or plasma components were capable of buffering the endotoxin released. The additional exposure of the supernatants to rabbit plasma reduced the amounts of free endotoxin to similar or lower than were those found after 12 hours of growth in its presence (Figure 3). Therefore, due to the high capacity of plasma to bind and neutralise endotoxin, the influence of the complement and other bactericidal plasma proteins on cell damage could not be objectively assessed in this study. This property of plasma could be assigned to different lipopolysacharides-binding proteins (Van Amersfoort et al., 2003).

CONCLUSION

In this study we have found a high variability among *P. multocida* rabbit isolates originating from different locations in the Czech Republic. PFGE has been shown to have a great discriminatory power in the typing of rabbit isolates and this method could be suggested for the epizootiological study of rabbit pasteurellosis. Fimbriae, which can play important role in colonisation of host tissue, were not found to be common among the Czech *P. multocida* rabbit isolates; however, this finding was based on the expression under *in vitro* conditions. Because clear haemolytic activity was observed in only the one isolate, it seems that haemolysis does not play a significant role in virulence of *P. multocida*, in general. Despite the bactericidal properties of plasma all isolates grew easily in its presence and the amounts of endotoxin were considerably lower than those found in the medium without plasma.

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