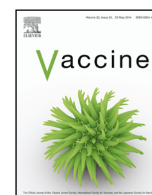
Vaccine 34 (2016) 1696–1703

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Protective efficacy afforded by live *Pasteurella multocida* vaccines in chickens is independent of lipopolysaccharide outer core structure

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ARTICLE INFO

Article history:
Received 26 November 2015
Received in revised form 1 February 2016
Accepted 3 February 2016
Available online 16 February 2016

Keywords:
Pasteurella multocida
Vaccine
Lipopolysaccharide

ABSTRACT

Pasteurella multocida is a major animal pathogen that causes a range of diseases including fowl cholera. *P. multocida* infections result in considerable losses to layer and breeder flocks in poultry industries worldwide. Both killed whole-cell and live-attenuated vaccines are available; these vaccines vary in their protective efficacy, particularly against heterologous strains. Moreover, until recently there was no knowledge of *P. multocida* LPS genetics and structure to determine precisely how LPS structure affects the protective capacity of these vaccines. In this study we show that defined lipopolysaccharide (LPS) mutants presented as killed whole-cell vaccines elicited solid protective immunity only against *P. multocida* challenge strains expressing highly similar or identical LPS structures. This finding indicates that vaccination of commercial flocks with *P. multocida* killed cell formulations will not protect against strains producing an LPS structure different to that produced by strains included in the vaccine formulation. Conversely, protective immunity conferred by vaccination with live *P. multocida* strains was found to be largely independent of LPS structure. Birds vaccinated with a range of live mutants belonging to the L1 and L3 LPS genotypes, each expressing a specific truncated LPS structure, were protected against challenge with the parent strain. Moreover, birds vaccinated with any of the five LPS mutants belonging to the L1 LPS genotype were also protected against challenge with an unrelated strain and two of the five groups vaccinated with live LPS mutants belonging to the L3 genotype were protected against challenge with an unrelated strain. In summary, vaccination with live *P. multocida aroA* mutants producing full-length L1 or L3 LPS or vaccination with live strains producing shortened L1 LPS elicited strong protective immunity against both homologous and heterologous challenge.

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1. Introduction

Pasteurella multocida is a Gram-negative pathogen of poultry and other production animals, and can also cause serious disease in humans [1]. Since 1972, Heddleston serotyping has been used to differentiate *P. multocida* strains into 16 serovars using antisera raised against the *P. multocida* somatic or lipopolysaccharide (LPS) antigens [2]. *P. multocida* LPS lacks an O-antigen and consists of lipid A, a highly conserved inner core region, and an outer core oligosaccharide comprising a variable number of specifically-linked sugars. Recent structural analysis has revealed that there are at least 22 outer core structures produced by *P. multocida* [1–8] and shown that the Heddleston system does not reflect the full LPS diversity in *P. multocida* and is not predictive of LPS structures expressed by different isolates [3]. To address the inaccuracies inherent in Heddleston serology we have developed an LPS-mPCR to accurately type *P. multocida* strains into one of eight LPS genotypes (L1 to L8) on the basis of the LPS outer core genetics [3].

It has been reported that *P. multocida* killed whole-cell vaccines elicit protective immunity only against strains belonging to the same Heddleston serovar [9]. Several manufacturers have developed live-attenuated vaccines and some level of heterologous protection has been reported [10]. However, as Heddleston typing is poorly predictive of LPS structure [3], it has previously not been

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http://dx.doi.org/10.1016/j.vaccine.2016.02.017
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Table 1
Bacterial strains and plasmids used in this study.

Strain, plasmid	Relevant description	Source or reference
Strains		
E. coli DH5α	*deoR*, *endA*1, *gyrA*96, *hsdR*17(r_k^- m_k^+), *recA*1, *relA*1, *supE*44, *thi*-1, (*lacZYA-argF*V169), Φ80*lacZ* ΔM15, F-	Bethesda Research Laboratories
P. multocida		
AL435	Fully virulent VP161 with single Tn*916* insertion in genome, Tet^R	[26]
AL523	AL435 *hptE* single cross-over mutant, Tet^R $Spec^R$	[14]
AL554	AL435 *gctB* single cross-over mutant, Tet^R $Spec^R$	[26]
AL571	AL435 *pcgC* single cross-over mutant, Tet^R $Spec^R$	[14]
AL725	AL435 *gatA* single cross-over mutant, Tet^R $Spec^R$	[14]
AL2116	P1059 *gatF* TargeTron® mutant, Kan^R	[11]
AL2117	P1059 *natB* TargeTron® mutant, Kan^R	[11]
AL2155	P1059 *gatG* TargeTron® mutant, KanR	[11]
AL2244	P1059 *aroA* double cross-over mutant	[27]
AL2453	VP161 *aroA* TargeTron® mutant, Kan^R	This study
AL2480	AL571 *aroA* TargeTron® mutant, Tet^R $Spec^R$ Kan^R	This study
AL2486	AL554 *aroA* TargeTron® mutant, Tet^R $Spec^R$ Kan^R	This study
AL2503	AL725 *aroA* TargeTron® mutant, Tet^R $Spec^R$ Kan^R	This study
AL2506	AL523 *aroA* TargeTron® mutant, Tet^R $Spec^R$ Kan^R	This study
AL2510	AL2244 *natB* TargeTron® mutant, Kan^R	This study
AL2513	AL2244 *gatF* TargeTron® mutant, Kan^R	This study
AL2517	AL2244 *gctC* TargeTron® mutant, Kan^R	This study
AL2519	AL2244 *gatG* TargeTron® mutant, Kan^R	This study
P1059	Heddleston serovar 3/LPS genotype L3 type strain	[28]
PM1422	Heddleston serovar 3/LPS genotype L3, virulent chicken isolate and challenge strain	This study
VP161	Heddleston serovar 1/LPS genotype L1, virulent chicken isolate and challenge strain	[29]
X73	Heddleston serovar 1/LPS genotype L1 type strain, virulent chicken isolate	[28]
Plasmids		
pAL953	*P. multocida* plasmid ($Spec^R$) containing TargeTron® group II intron (kan^R)	[11]
pAL1003	pAL953 retargeted to *natB*	[11]
pAL1004	pAL953 retargeted to *gatG*	[11]
pAL1006	pAL953 retargeted to *gatF*	[11]
pAL1081	pAL953 retargeted to *aroA* using primers BAP7156, BAP7157 and BAP7158	This study
pAL1106	pAL953 retargeted to *gctC* using primers BAP7299, BAP7300 and BAP7301	This study

possible to accurately test the breadth of homologous and heterologous protection afforded by *P. multocida* vaccines. Furthermore, as no-one has had access to isogenic strains expressing different LPS structures, the role of LPS structure in vaccine protective efficacy has never been objectively assessed. In this study we have used our knowledge of the *P. multocida* LPS genetics and structure to precisely determine how LPS structure affects vaccine efficacy and to objectively compare the protective capacity of killed whole-cell and live-attenuated *P. multocida* vaccines.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. In this study, *P. multocida* strains belonging to the L1 (strains VP161, X73) and L3 (strain P1059) LPS genotypes were selected as vaccine strains as they produce distinct LPS outer core structures and PCR analysis revealed that the majority (>75%) of Australian field isolates belong to these two LPS genotypes [3]. *Escherichia coli* and *P. multocida* strains were grown routinely in Luria-Bertani broth and heart infusion (HI) broth, respectively (Oxoid, Basingstoke, United Kingdom); solid media were obtained by the addition of 1.5% agar. For antibiotic selection, spectinomycin (100 μg/ml), kanamycin (50 μg/ml) or tetracycline (2.5 μg/ml) was added to the media (where appropriate). For *in vitro* growth of *aroA* mutants, media were supplemented with an aromatic acid mix containing tryptophan, phenylalanine (both at final concentration of 40 pg/ml), 2,3-dihydroxybenzoic acid and *p*-hydroxybenzoic acid (both at final concentration of 10 pg/ml). For vaccine and challenge strain preparation, *P. multocida* strains were grown overnight in 10 ml of HI broth (with aromatic acid supplementation where required) and the appropriate antibiotic. Each culture was then subcultured into 10 ml of HI media (plus aromatic acid supplementation for *aroA* mutants) containing no antibiotics and grown at 37 °C with shaking until mid-exponential phase of growth was attained ($O.D_{600\,nm}$ = 0.45). Bacterial cell numbers were determined by standard viable count.

2.2. DNA manipulations

Restriction digests and ligations were performed according to the manufacturers' instructions using enzymes obtained from NEB (Ipswich, MA) or Roche Diagnostics (Mannheim, Germany). Plasmid and genomic DNA was prepared using the Plasmid Mini kit from QIAGEN (Hamburg, Germany) and the Genomic DNA extraction kit from RBC (Banqiao City, Taiwan), respectively. DNA was PCR amplified using Taq DNA polymerase (Roche Diagnostics) and purified using the Qiaquick PCR Purification Kit (QIAGEN, Germany). Oligonucleotides (Supplementary Table 1) were synthesized by Sigma, Australia. Sequencing reactions were performed using PRISM BigDye Terminator Mix version 3.1 (Life Technologies, Carlsbad, CA) as described previously [11]; DNA sequences were determined on a capillary-platform Genetic Analyser (Applied Biosystems 3730) and analysed with Vector NTI Advance 11 (Life Technologies, Carlsbad, CA). Gene-specific intron insertion into *P. multocida* was conducted using TargeTron technology (Sigma-Aldrich, St. Louis, MO) and mutagenesis of *P. multocida* was performed as described previously [11,12]. Each mutant was checked by PCR and direct sequencing from genomic DNA was performed to confirm single intron integration into the correct target gene as described previously [11]. The relative size of the LPS expressed by each *aroA*/LPS double mutant was directly compared to those of LPS mutants, for which the full LPS structure has been determined [3], using polyacrylamide gel electrophoresis (PAGE) and carbohydrate silver stain (data not shown) as described previously [13].

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