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Using Red Recombinase and PCR Product for One-Step in-Frame Inactivation of *aroA* Gene in *Pasteurella multocida* A: 1 and Evaluation of Pathogenicity and Immunogenicity

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Abstract: An *aroA* mutant of a local strain of *Pasteurella multocida* A: 1 was produced using Red recombinase and PCR product. A pair of primers (aroAPmF and aroAPmR) were used that contained sequences homologous to regions adjacent to the gene to be inactivated followed by sequence of a flanking FRT site. A PCR process with these primers and the template plasmid pKD4 containing a kanamycin resistance gene with FRT (FLP recognition site) flanks was performed and the purified product (~1.6 kb) was transformed into the wild type strain which had already been transformed with pKD 46, a plasmid containing λ Red recombinase gene. The transformed product replaced the original gene by homologous recombination. This method does not need cloning or sequencing of the gene, any restriction enzyme cuts or special shuttle or suicide vectors. The mutant strain was selected on media containing kanamycin and verified with PCR using both pairs of primers (AroAPmF and AroAPmR, AroA1F and AroA2R), then tested for attenuation and immunity induction by challenge in chickens, as mutation in the *aroA* gene creates dependency for growth on aromatic compounds that are not available in host tissues and the *aroA* mutants of *P. multocida* would provide good protection against challenge in chickens.

Key words: *Pasteurella multocida*, *aroA*, red recombinase, PCR product

INTRODUCTION

Pasteurella multocida is recognised as important pathogen and causative agent of the disease known as pasteurellosis in different animal species worldwide. *P. multocida* of capsular type A can cause severe forms of avian pasteurellosis or fowl cholera with clinical signs of pneumonia, tracheitis, hemorrhagic septicemia, hepatitis and high mortality rates (Rhoades and Rimler, 1989; Scott *et al*., 1999). Some strains have been isolated from outbreaks of fowl cholera in birds (Tavasoli *et al*., 1984) and capsular group and somatic type has been determined as serotype A: 1 (Sotoodenia *et al*., 1986). The isolates are known to be highly virulent for chickens, by intramuscular inoculation of even 7 CFU of the strain (Sotoodenia *et al*., 2004). They are used to prepare bacterins as killed vaccine.

There is a need in the commercial poultry industry for safe, live attenuated fowl cholera vaccines that provide long term and reliable protection (Scott *et al*., 1999). Such vaccines are more reliable when derived from local pathogens to provide similar antigenic properties. Some auxotrophic mutants of *P. multocida* have been developed by creating non-reverting mutations in the *aroA* gene that render them growth dependent on final products of the aromatic biosynthetic pathway, including particular amino acids not available in host tissues. Mutations have been performed to inactivate the gene by cloning the gene in a shuttle vector, insertion of an antibiotic cassette as marker (Homchampa *et al*., 1992) or deletion of an internal sequence of the gene (Homchampa *et al*., 1997) using restriction enzymes and subsequently homologous recombination using a suicide vector. The mutants have been well protective. The method has been used to construct similar mutants of *P. multocida* serotype B: 2 (Tabatabaei *et al*., 2002) and *Pasteurella (Mannnheimia) haemolytica* serotype A:1 (Tatum *et al*., 1994). Some new shuttle vectors have been developed (Briggs and Tatum, 2005) and used to construct in-frame *aro*A deletion mutants of *M. hemolytica, P. multocida* and *Haemophilus somnus* (Tatum and Briggs, 2005). Studies have been done to

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improve the tools and systems and to solve the problems of multiple steps in the production of such mutants. Although, a number of general allele replacement methods can be used to inactivate bacterial chromosomal genes, these all require creating the gene disruption on a suitable plasmid before recombining it onto the chromosome. Kim *et al.* (2006) have inserted a kanamycin gene into amplified and cloned *toxA* gene using restriction enzyme digestion and replaced it by the original gene in *P. multocida* containing pKD46 which produces λ Red recombinase. A simple and highly efficient method for one-step disruption and inactivation of chromosomal genes in *E. coli* K-12 using PCR products has been developed (Datsenko and Wanner, 2000). This method does not need cloning or sequencing of the gene, any restriction enzyme cuts or special shuttle or suicide vectors.

The aim of the present study was to make one-step in frame mutation in the *aroA* gene of a local strain of *P. multocida* A: 1 using red recombinase and PCR products to set up an easy and efficient approach to construct various *Pasteurella* mutants and attenuated strains.

MATERIALS AND METHODS

Media, chemicals and other reagents: Culture media, chemicals and reagents were prepared from Merck (Germany) unless indicated otherwise. Ampicillin (Ap^R) and kanamycin resistant (Km^R) transformants were selected and cultured on 5% sheep blood agar or brain heart infusion agar and broth containing the respective antibiotics at 100 and 50 μg mL^{-1}. Pathogenic *P. multocida* serotype A: 1 isolated from a case of acute fowl cholera was used as wild type strain to be manipulated. Extraction of plasmids (Mini-preps alkaline lysis) and genomic DNA were performed according to Sambrook *et al.* (2000). Fermentas (Canada) products were used for PCR amplification of the gene (Taq DNA polymerase for all reactions), agarose gel electrophoresis, extraction and purification (DNA extraction kit) and cloning (InsT/Aclone™ PCR product cloning kit). Plasmids pKD46 and pKD4 were kindly donated by Dr. Ivan Rychlik (Vet. Res. Inst., Brno, Czech Rep.). Competent cells of *P. multocida* were prepared according to Kim *et al.* (2006). Transformations were performed by electroporation using gene pulser system of Bio-Rad (UK) according to Tabatabaei *et al.* (2002).

PCR process and recombination: The *aroA* gene of *P. multocida* A: 1 was amplified as a ~1.2 kb fragment using primers AroA1F (5'-TTA CTC TCA ATC CCA TCA GC-3') and AroA2R (5'-ACA ATG CGA TTA AAG CAA AG-3') (Homchampa *et al.*, 1992; Tabatabaei *et al.*, 2002) (gene bank accession number Z14100) as follows: 30 cycles of 92°C for 40 sec, 55°C for 45 sec and 72°C for 55 sec, followed by a final extension of 72°C for 25 min. The purified PCR product of *aroA* gene was ligated with pTZ57R/T (2886 bp). The new plasmid (pPMZA1) was transformed into *E. coli* DH5-α using InsT/Aclone™ PCR product cloning kit (Fermentas, Canada) for sequencing and further studies. A pair of primers AroAPmF (5'-ATG GAA TCC CTG ACG TTA CAA CCC ATC GCG CGG GTC GAT GGC GCG TGT AGG CTG GAG CTG CTT C-3') and AroAPmR (3'-TTA GGC AGG CGT ACT CAT TCG CGC CAG TTG TTC GAA ATA ATC AGC ATA TGA ATA TCC TCC TTA G-5') were designed according to the nucleotide sequence in the GenBank database for the *aroA* gene and adjacent genes of *P. multocida* A:1 (Homchampa *et al.*, 1992; May *et al.*, 2001) (44 first nucleotides) followed by 20 nucleotides homologous to flanking FRT site on pKD4. Plasmid pKD4 contains kanamycin resistance gene flanked by FRT recognition site. These primers were used for a PCR with 30 cycles of 92°C for 40 sec, 55°C for 45 sec and 72°C for 55 sec, followed by a final extension of 72° C for 3 min with pKD4 as template DNA. The product would be a ~1.6 kb fragment which contains ends homologous to *aroA* gene ends, two flanking FRT recognition sites and a central kanamycin resistance gene (Datsenko and Wanner, 2000). This fragment would replace the original gene by homologous recombination and cause the mutation of the transformed strain in the replaced gene.

A Red helper plasmid pKD46 (Datsenko and Wanner, 2000), which uses the pBAD promoter to express red and gam from a low copy number temperature-sensitive replicon was used. This plasmid contains λ Red recombinase, which allows recombination in linear DNA in bacteria to occur successfully and is a temperature sensitive replicon to allow for its easy elimination at 43°C. The plasmid also contains ampicillin resistance gene as marker for selection. The plasmid was transformed to competent cells of wild type *P. multocida* A: 1 by electroporation and one successful transformant was selected on ampicillin-containing sheep blood agar.

The PCR product of ~1.6 kb size was purified and transformed in the pKD46-containing *P. multocida* A: 1 by electroporation according to Kim *et al.* (2006). After selection of transformed strain (pumA) on kanamycin-containing sheep blood agar, using its purified genomic DNA as template a similar PCR was performed with primers AroAPmF and AroAPmR, or AroA1F and AroA2R to verify successful recombination and replacement.

Virulence and protection test: A 10-days-old Ross broiler chickens from a *P. multocida* free flock were used for challenge to determine the virulence, attenuation and immunity induction of wild type and mutant strains according to guidelines of OIE (1996) manual for avian pasteurellosis and Scott *et al*. (1999). Six groups of 12 healthy chickens from both sexes were isolated in and injected intramuscularly (Scott *et al*.,1999) with 0.5 mL of 10 fold serially diluted in PBS of exponential phase culture grown in BHI broth. They propose that 10^4 CFU of attenuated *aroA* mutant of avian *P. multocida* would be protective and safe. Two more concentrations were also added. Treatments were as follows: Group A (10^8 CFU pumA), group B (10^7 CFU pumA), group C (10^6 CFU pumA), group D (10^2 CFU wild type), group E (10 CFU wild type) and group F (negative control with sterile medium). CFU counts have been confirmed by plate counting method. Chickens were monitored and examined at regular intervals after challenge. One bird from each group at days 2 and 7 after vaccination plus those showing any clinical signs of disease (ruffled feathers, loss of appetite...) were euthanized and necropsied for the observation of postmortem signs and sampling for microbiological studies. For collection of blood samples and internal organs, chickens were sacrificed and samples were collected aseptically. First of all, impression smears from different internal organs such as liver, lung and blood smears from heart blood were prepared, fixed and stained by routine Gram staining. Organs including heart, lung, liver, tibial bone marrow and injected muscle were homogenized in 10 mL PBS and 100 μL aliquots of 10-fold serial dilution was plated on sheep blood agar and incubated overnight at 37°C for bacterial isolation.

For protection test, 14 days after first challenge, healthy chickens in groups A, B, C (inoculated with mutant strain) and F were challenged with 10^2 CFU of wild type *P. multocida* A: 1 strain. Chickens were monitored for an additional 2 weeks and then euthanized for further studies with the same method. In all steps, chickens were treated in accordance with animal ethics guidelines.

RESULTS

Construction of mutant strain: The *aroA* gene (~1.2 kb) was amplified from wild type strain by PCR, purified and ligated in pTZ57R/T (2886 bp). The PCR with primers AroAPmF and AroAPmR and pKD4 as template produced a fragment of ~1.6 kb size. Competent cells of *P. multocida* A: 1 wild type strain which did not contain any original plasmid were successfully transformed with pKD46 by electroporation. Transformed strain was selected and isolated on ampicillin-containing sheep blood agar incubated at 30°C. Extraction of plasmid DNA and checking by agarose gel electrophoresis confirmed its presence in the strain. This strain was transformed with gel-purified ~1.6 kb PCR product. The successful recombinant strain was selected by culture on kanamycin-containing sheep blood agar incubated at 37°C. Two more similar subcultures were repeated to cure the recombinant strain from pKD46. The success of recombination and mutation was verified by PCR with extracted genomic DNA of pumA strain as template. Primers AroA1F and AroA2R amplified the original gene (~1.2 kb) in wild type strain but could not amplify any product in pumA. Primers AroApmF and AroAPmR could amplify ~1.6 kb fragment in pumA but did not amplify any sequence in wild type strain (Fig. 1).

Virulence and protection test: All chickens receiving wild type strain showed clinical signs of fowl cholera 24 h post challenge and wild type *P. multocida* was isolated from blood, lung, liver, bone marrow and injected muscle. Chickens of all other groups which received pumA and negative control group did not show any clinical signs of

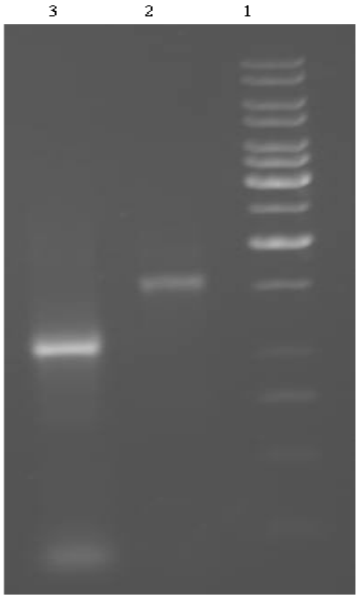

Fig. 1: The PCR products with genomic DNAs of wild type and *aroA* mutant of *P.multocida* A: 1 as templates. Lane 1: 1 kb size marker. Lane 2: The PCR product (~1.6kb) amplified with AroApmF and AroApmR primers from genomic DNA template of *P. multocida* A: 1 *aroA* mutant (replaced *aroA* gene in genomic DNA). Lane 3: The *aroA* gene (~1.2kb) amplified with AroA1F and AroA2R primers from genomic DNA template of wild type *P. multocida* A: 1

disease and no *P. multocida* could be isolated from their blood, lung, liver, bone marrow or injected muscle. Chickens receiving puma were completely protected and immunized against challenge with wild type strain and no clinical signs could be observed after wild type challenge. No mutant or wild type *P. multocida* could be isolated from their blood, lung, liver, bone marrow or injected muscle.

DISCUSSION

Different methods for induction of mutation in bacteria have been introduced and one of the most favorable approaches is in-frame mutation performed by homologous recombination and replacement of the original gene by an enzymatically disrupted gene ligated and cloned in a vector. This approach is time-consuming and needs shuttle and suicide vectors able to be active in the target strain. Additionally, it needs restriction enzymes to cut specific sites on the gene to be disrupted and not active on the sequence of shuttle plasmids and marker insert gene. The use of red recombinase-containing plasmids like pKD46 solves the need for ligation in a suicide vectors by increasing the rate of recombination in a simple transformation of replacing gene. This plasmid has been used for swine *P. multocida* to replace an enzymatically disrupted product by *toxA* gene (Kim *et al*., 2006). We could easily transform pKD46 into the target wild type avian *P. multocida* A: 1 by electroporation. The plasmid could survive and multiply at 30°C and was easily cured by incubation at 37°C. The genes disrupted by enzyme digestion and inserted with antibiotic resistance gene usually have much larger size (sometimes twice) than the original gene (Homchampa *et al*., 1992; Tabatabaei *et al*., 2002) which can be troublesome in homologous recombination. Our PCR product had a size close to original gene (~1.6 and ~1.2 kb, respectively). The PCR product containing kanamycin gene could be transformed into the competent cells and replaced the original gene by homologous recombination. One PCR set could show the difference in the size and sequence of the gene in the wild type and mutant strains. There is no need to know the exact sequence and restriction sites of any gene or plasmids. Similar work has been done on *E. coli* K-12 using PCR products (Datsenko and Wanner, 2000). This method can be used to delete every known gene in the genomic map of *P. multocida* as it just needs access to total genomic map and not the exact sequence of the gene for restriction digestion. There is even possibility to delete more than gene with adjacent or far arrangement in genome. Deletion of a set of djacent genes can be done using just one PCR product containing just both ends of the fragment. *P. multocida* with limited information about their function and this method can be easily used to study the behavior and phenotype of the specific gene-knock out mutants (May *et al*., 2001). The antibiotic resistance gene flanked by FRT recognition site can be deleted from the genome after recombination using the plasmid pCP20 (Datsenko and Wanner, 2000). This needs the transformation of the plasmid into the mutant strain and subculture at 30°C which has not been performed yet.

Use of live attenuated strains which can be easily produced from field isolates and have good immunogenic properties is a real demand and *aroA* mutants have proven to be good attenuated immunogenic strains (Homchampa *et al*., 1992; Scott *et al*., 1999). Our *aroA* mutant was completely attenuated and showed acceptable immunogenic properties and protection without any adverse effects in vaccinated challenged chicken hosts. Similar protection studies have indicated that 10^6 CFU of candidate vaccine strain would be suitable (Scott *et al*., 1999). In the present study, three doses 10^6, 10^7 and 10^8 CFU of the attenuated strain were safe and could induce protective immunity against the following wild type challenge although the mutant strain did not survive in the host tissues for a long time and could not be isolated 48 h after injection.

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