

## DNA Fingerprinting Analysis of Breakthrough Outbreaks in Vaccine-Protected Poultry Stocks<sup>▽</sup>

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**We report recurrent outbreaks of *Yersinia pseudotuberculosis* conjunctivitis in ducks and of fowl cholera in geese, occurring in stocks previously vaccinated with inactivated autogenous vaccines. Enterobacterial repetitive intergenic consensus sequence-based PCR and pulsed-field gel electrophoresis indicated reinfection with a new *Y. pseudotuberculosis* strain and vaccine evasion by the same *Pasteurella multocida* strain.**

Vaccination is the most effective approach in the prevention and control of various human and veterinary infectious diseases. Two major problems that may corrupt the efficiency of vaccines are reversion of attenuated organisms and loss of protective effect. The latter may be due either to reinfection or superinfection with another strain against which the vaccine does not protect (4) or to evolution of the original strain to evade the immune response elicited by the vaccine (5, 10, 14).

The two major causes of loss of vaccine protection pose a diagnostic challenge and necessitate different epidemiological countermeasures besides development of new vaccines. In the case of reinfection, the novel infectious source must be discovered and eliminated, while in the case of vaccine reversion and pathogen evolution, the vaccination strategy should be revised and improved to prevent persistence, which would prevent the pathogen from evolving under the vaccine-exerted selective pressure. Therefore, it can be important to discover which of the two causes lies in the background of a particular breakthrough disease to optimize countermeasures.

To achieve this aim, ideally, the relationship of the original strains, the vaccine strain and the strain causing the breakthrough disease, should be explored. However, in some cases, the vaccine strain is not available (due to, for example, confidentiality reasons). In these cases, conclusions can be drawn only from a comparison of the prevaccination and breakthrough strains. Molecular epidemiology methods proved very useful in such investigations (15).

Similar cases of follow-up when a vaccine became ineffective have been documented for animals with viral diseases caused by porcine respiratory and reproductive syndrome virus (8, 12) and avian metapneumovirus (2), as well as for patients infected with *Bordetella pertussis* (10, 14) and animals with swine erysipelas (3, 6).

This work reports two cases of breakthrough infection in

vaccinated poultry stocks analyzed by DNA fingerprinting methods.

Outbreak series 1 involved a Barbary duck breeding stock affected by recurring epizootics of severe conjunctivitis. As import animals were regularly introduced into the stock to avoid inbreeding and as a similar disease was observed in the stock of their origin, the disease was considered imported. It affected almost all animals and manifested itself at the end of the laying period or after molting, causing only a few fatalities but leading to significant deterioration. The first outbreak occurred in 1998 after the ducks molted. While the disease could be partially suppressed by oxytetracycline treatment, the pathogen could not be eradicated, and the disease recurred regularly. Conjunctival samples collected in the field and in the laboratory from live animals affected by the two studied outbreaks yielded *Yersinia pseudotuberculosis*. Using one of the isolates, a stock-specific inactivated vaccine was developed by serial passages in artificial media. This vaccine was used regularly, and the outbreaks ceased. In March 2005, a breakthrough outbreak occurred in spite of the unchanged vaccination protocol. The timing of and symptoms exhibited during the outbreak were identical to these characteristics for the outbreak of 1998. Vaccination was continued, but two similar outbreaks followed. Diagnostic procedures identical to those applied earlier demonstrated *Y. pseudotuberculosis* isolates from all three outbreaks.

In outbreak series 2, fowl cholera occurred in multiple large (over 10,000 animals) goose flocks of a large-scale breeder in 2003. Animals died with acute septicemia, and upon necropsy, abdominal hyperemia with subserosal hemorrhages was discovered. Cultures from liver, spleen, and/or blood of dead or moribund animals yielded *Pasteurella multocida*. After the cessation of outbreaks, vaccination with inactivated vaccine was initiated to prevent recurrences. The applied vaccine contained two strains that protect against the two serotypes most common in Hungarian fowl cholera (A1 and A3) plus the strain that caused the outbreaks in the flocks (autogenous vaccine). This vaccine protected the flocks from fowl cholera until 2006, when high-mortality outbreaks occurred again, affecting almost all premises of the company, which are situated relatively far from each other. Sporadic cases kept occurring for months

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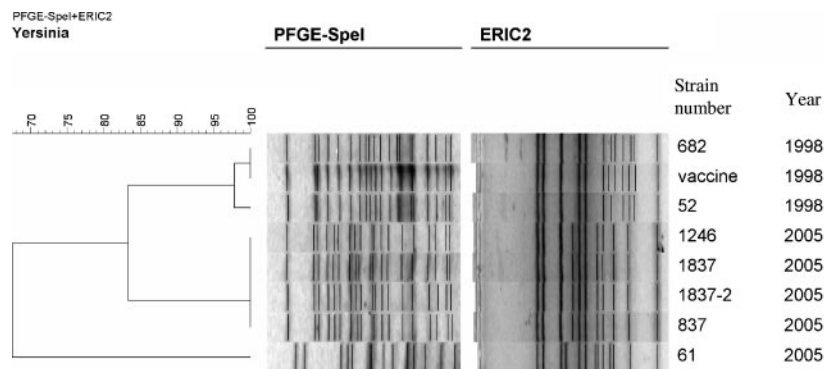


FIG. 1. Composite dendrogram generated from data for PFGE and ERIC-PCR profiles of *Y. pseudotuberculosis* strains. Strain 1837 was tested twice in separate experiments to ensure reproducibility. Strain 61 is the outgroup isolate.

after the cessation of outbreaks. The plucker team is suspected to have mediated the transmission of pasteurellae among the premises.

Six *Y. pseudotuberculosis* isolates associated with outbreak series 1 were studied. Two and three strains were isolated from two and three different outbreaks in 1998 and 2005, respectively, and the vaccine strain based on the strain from the second outbreak in 1998 was also examined. One of the isolates from 2005 was tested in duplicate from different frozen stocks to confirm reproducibility. A *Y. pseudotuberculosis* isolate originating from a different area was used as an outgroup isolate.

From outbreak series 2, two isolates from 2003 and 19 from 2006 were studied. The vaccine strains were not available. Several isolates of *P. multocida* from animals affected by fowl cholera outbreaks occurring in different stocks and different species were used as outgroup isolates, including the strains, found to be unrelated, isolated from two fowl cholera outbreak series reported earlier (7). All outgroup isolates were recovered between 1998 and 2006 from fowl cholera outbreaks oc-

curing in the same region. All isolates were stored as a frozen or lyophilized stock until testing.

DNA was extracted from cultures by boiling a loopful of bacteria at 98°C for 15 min. The capsular serotype of *P. multocida* was determined using the multiplex PCR described by Townsend et al. (13). The genetic relatedness of isolates from both outbreak series was studied by using an enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) assay performed with primers and PCR conditions described earlier (1) and using pulsed-field gel electrophoresis (PFGE). These methods were demonstrated to be sufficiently discriminatory for *P. multocida* (9) and *Y. enterocolitica* (11), a pathogen closely related to *Y. pseudotuberculosis*. Plugs for PFGE were prepared as described earlier (7) and digested with SpeI (Promega) for *Y. pseudotuberculosis* or ApaI (Promega) for *P. multocida* by following the manufacturer's instructions. PFGE was performed in a CHEF-DRIII apparatus (Bio-Rad), in 1% SeaKem Gold agarose (Cambrex) at 14°C, using 6 V/cm with a reorientation angle of 120°. Switch times were increased from 2 to 64 s for 22 h for *Y. pseudotuberculosis* and from 5 to

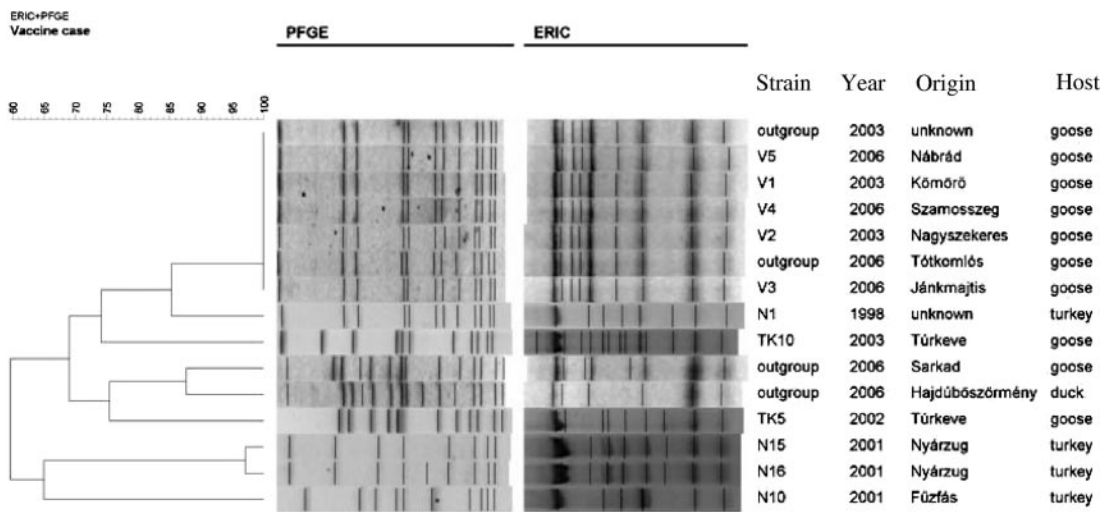


FIG. 2. Composite dendrogram generated from data for PFGE and ERIC-PCR profiles of *P. multocida* strains. V1 through V5 are the first five strains from outbreak series 2. The profiles of the other 14 strains were identical, and they clustered together with V1 through V5 (data not shown). N1, N10, N15, N16, TK5, and TK10 are strains from the two outbreaks reported earlier (see reference 7; strain numbers are identical to those used therein).

30 s for 15 h and then from 30 to 90 s for 7 h for *P. multocida*. Banding patterns were analyzed with the software Fingerprinting II (Bio-Rad), using the Dice coefficient and the unweighted-pair group method with averages for the generation of dendrograms.

As shown in Fig. 1, *Y. pseudotuberculosis* isolates formed two clusters segregated according to the year of isolation. Expectedly, the vaccine strain was indistinguishable from the second outbreak isolate of 1998, from which it was derived, and the outgroup isolate proved to be clearly distinct by both ERIC-PCR and PFGE. These results show that outbreaks occurring before the initiation of vaccination were caused by the same persisting clone, which caused disease in the stock when immunity was weakened during molting or by the end of the laying period. This strain could be controlled by the vaccine. The outbreaks in 2005 were caused by a similarly persisting clone, against which the vaccine that was used did not seem to protect appropriately. Similarity data suggest that, presumably, this strain was a novel one imported and introduced to the stock in 2005, but the possibility of microevolution of the original strain could not be ruled out unequivocally.

All *P. multocida* isolates from outbreak series 2 belonged to serogroup A, and both fingerprinting methods grouped together the successive isolates (Fig. 2). All of these isolates showed 100% similarity with both methods, regardless of year or site of isolation. Interestingly, two independent outgroup isolates proved to be indistinguishable from isolates of the outbreak-related cluster, suggesting that these isolates may belong to a particularly successful strain widespread in eastern Hungary. Isolates representing different strains from two outbreak series reported earlier (7) proved to be distinct from the strain causing the breakthrough outbreak presented here. Persistence of the original outbreak strain suggests that the vaccine lost its protective power, even though the vaccine strain could not be investigated. Three explanations are possible. The first explanation is that the affected stocks suffered unusual stress, leading to increased susceptibility to infection. This is contradicted by the successive spread of pasteurellae to stocks situated far from each other and by the continued occurrences of sporadic cases. The second explanation is that breakthrough outbreaks were due to some unknown deficiency in the vaccination protocol or in the particular vaccine stock used in 2006. The third explanation is that vaccine evasion was due to microevolution of the strain, leading to increased virulence or to changes in surface antigen expression. At the same time, similarly to the results of our earlier work (7), these results draw attention to the danger of persistence and exchange of pasteurellae among flocks of large-scale poultry breeders.

DNA fingerprinting revealed equally important but different data in the two outbreaks. In outbreak series 1, reinfection with a new strain is suspected, necessitating careful source-tracking work. As the reinfection was presumably due to a new importation event in this particular case, the only remedy possible was a vaccine with the novel strain included, which proved to be successful. However, to prevent repetition of importation,

careful quarantining and a meticulous search for yersiniae in imported animals are advisable.

In outbreak series 2, the outbreak strain was demonstrated to persist in spite of vaccination and to cause disease after being suppressed for years. Fingerprinting data point to evasion of the immune response, an outcome which warrants, besides the introduction of stricter infection control measures, a revision of the vaccination protocol.

These two outbreak series demonstrate the usefulness of DNA fingerprinting in investigating the cause of a loss of vaccine protection by tracing strains responsible for the outbreaks. This approach may provide further information to add to the findings obtained by vaccine efficacy studies. Furthermore, it can be used even when the vaccine strain is not available for study, as this approach provides clues necessary for proper countermeasures and thus may save time, money, and valuable animals.

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