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Use of an Electronic Nose To Diagnose *Mycobacterium bovis* Infection in Badgers and Cattle

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It is estimated that more than 50 million cattle are infected with *Mycobacterium bovis* worldwide, resulting in severe economic losses. Current diagnosis of tuberculosis (TB) in cattle relies on tuberculin skin testing, and when combined with the slaughter of test-positive animals, it has significantly reduced the incidence of bovine TB. The failure to eradicate bovine TB in Great Britain has been attributed in part to a reservoir of the infection in badgers (*Meles meles*). Accurate and reliable diagnosis of infection is the cornerstone of TB control. Bacteriological diagnosis has these characteristics, but only with samples collected postmortem. Unlike significant wild animal reservoirs of *M. bovis* that are considered pests in other countries, such as the brushtail possum (*Trichosurus vulpecula*) in New Zealand, the badger and its sett are protected under United Kingdom legislation (The Protection of Badgers Act 1992). Therefore, an accurate in vitro test for badgers is needed urgently to determine the extent of the reservoir of infection cheaply and without destroying badgers. For cattle, a rapid on-farm test to complement the existing tests (the skin test and gamma interferon assay) would be highly desirable. To this end, we have investigated the potential of an electronic nose (EN) to diagnose infection of cattle or badgers with *M. bovis*, using a serum sample. Samples were obtained from both experimentally infected badgers and cattle, as well as naturally infected badgers. Without exception, the EN was able to discriminate infected animals from controls as early as 3 weeks after infection with *M. bovis*, the earliest time point examined postchallenge. The EN approach described here is a straightforward alternative to conventional methods of TB diagnosis, and it offers considerable potential as a sensitive, rapid, and cost-effective means of diagnosing *M. bovis* infection in cattle and badgers.

Accurate and reliable diagnosis of infection with members of the *Mycobacterium tuberculosis* complex is the cornerstone of tuberculosis (TB) control, whether in humans, cattle, or other species. It is estimated that, worldwide, there are more than 50 million cattle infected with *Mycobacterium bovis*, resulting in economic losses of approximately $3 billion annually (49). Current diagnosis of TB in cattle relies on tuberculin skin testing, the detection of a delayed-type hypersensitivity reaction to the intradermal inoculation of tuberculin. An enzyme-linked immunosorbent assay for detecting the release of gamma interferon in whole blood stimulated with tuberculin was developed in the early 1990s (47) and is now being used in some countries (such as New Zealand and the Republic of Ireland) as an ancillary test in particular situations, e.g., for herds with severe or chronic outbreaks of bovine TB. Combined with the slaughter of test-positive animals and control of the movement of cattle, the skin test has been instrumental in eradicating TB from some countries (e.g., Australia) and in reducing the incidence of bovine TB in many others. However, in situations in which there is a wildlife reservoir of infection, such as with badgers in Great Britain and the Republic of Ireland and with brushtail possums in New Zealand, this strategy has failed to eradicate the disease. Indeed, in Great Britain over the last two decades, there has been a dramatic rise in the incidence of TB in cattle (28).

Accurate diagnosis of *M. bovis* infection in badgers can be achieved by bacteriological examination but only for postmortem samples. Therefore, accurate in vitro diagnosis is required to allow disease surveillance of the badger population, as well as to underpin any possible future policies to control TB in badgers, for example, by vaccination. However, the best of the antibody assays for TB in badgers is only 62% sensitive (27), whereas in vitro cellular assays are better but impractical for routine use (8). In addition, a rapid on-farm test for cattle, complementing existing tests, could be beneficial.

Against this background, we have investigated the potential of an "electronic nose" (EN) to diagnose infection of cattle or badgers with *M. bovis* by using a serum sample. Smell can be used to diagnose diseases and has been used by both the Greeks and the Chinese since 2000 BC (reviewed in references 41 and 54). Electronic nose is the colloquial name for an instrument made up of chemical sensors combined with a pattern recognition system (18). The key function of an EN is to mimic the human olfactory system. In the EN, the human olfactory receptors have their analogues in chemical sensors

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that produce an electrical signal (similar to nerve cells). These signals are subsequently analyzed by pattern recognition software. The pattern recognition software corresponds to the cerebral cortex of the brain and is able to classify and memorize odors (3, 42, 43).

The most commonly used types of sensors in ENs are metal oxide sensors (10, 45), conducting polymers (17, 23), and piezoelectric-based sensors, such as bulk acoustic wave sensors or surface acoustic wave sensors (7, 9). The sensors are characterized by a partial specificity, i.e., they respond to a certain group of chemicals, such as alcohols and aldehydes, etc., rather than a single compound (18). This partial specificity generates a unique signature (pattern) of the sample. All types of sensors share a common basic principle: the interaction of volatile compounds with the sensor surface leads to a change of physical properties (conductivity, resistance, and frequency) of the sensor, which is measured.

The EN has been applied in several areas to characterize the odors of products such as wine (11), beer (44), and paper (25). More recently, ENs have been used for the quality control and process monitoring of foodstuffs such as olive oil (20, 21) and milk (32). However, the potential of the EN as a diagnostic tool is attracting an increasing number of research groups for the diagnosis of infectious diseases such as bacterial vaginosis (6) and pulmonary (29) and urinary tract infections (1, 39), as well as breath analyses of patients suffering from diabetes (53), uremia (30), or lung cancer (12). Recently, the approach was used to diagnose the causative organism of diarrhea by sniffing stools (46), demonstrating the versatility of the approach. The present study demonstrates that EN technology can also be used for the diagnosis of TB in both cattle and badgers and is the first report of the application of EN sensing to serum.

MATERIALS AND METHODS

Animals and serum samples. Sera from badgers (*Meles meles*) were obtained from three sources. In the first case, badgers were experimentally infected with *M. bovis* via the endobronchial route at three different doses: 10, 100, and 3,000 CFU. These doses were chosen to generate a lung infection that would mimic that seen in naturally infected badgers. Three badgers were used for each dose, and an additional three unchallenged animals were used as controls. Blood was obtained from each animal 3 weeks prior to challenge, immediately prior to challenge, and at three weekly intervals after challenge for 15 weeks.

In the second case, 12 badgers were experimentally infected with 10,000 CFU of *M. bovis* via the endobronchial route. This dose was chosen to provide a reproducible pattern of disease in all inoculated badgers. An additional four unchallenged animals served as controls. Three experimentally infected badgers were euthanized at 6, 12, and 18 weeks after infection for other purposes, with the remaining badgers being euthanized at week 24. Blood was obtained from each animal 3 weeks prior to challenge, immediately prior to challenge, and at three weekly intervals after challenge for the duration of the experiment.

All badgers recruited for the experimental studies originated from locations in the Republic of Ireland where the badger population was known to be free of TB. The badgers were housed in a facility divided into enclosures equipped with artificial setts. After experimental infection, all badgers were confirmed to be positive for *M. bovis* infection by culture from tissue samples postmortem. Uninfected control animals were similarly confirmed to be culture negative. Full details of the experimental challenge studies will be submitted for publication elsewhere.

The final sources of badger sera were 19 animals that had been killed as part of the Department of Environment Food and Rural Affairs/Independent Scientific Group Randomised Badger Culling Trial (13). Each badger was subjected to routine postmortem examination and culture for the presence of *M. bovis*. Sera were chosen for study from 10 culture-confirmed tuberculous badgers and 9 culture-negative badgers.

Cattle sera were obtained from animals experimentally infected with *M. bovis*. Eight calves (4- to 5-month-old female Holstein-Friesians) were infected with an *M. bovis* field strain from Great Britain (AF 2122/97) by endobronchial instillation of 4×10^4 or 6×10^4 CFU as described previously (52). Blood samples were collected from all animals 3, 5, 8, and 15 weeks after challenge and from six of the calves at 24 weeks. Animals were skin tested with the single intradermal comparative cervical tuberculin test 14 weeks after *M. bovis* infection. Two animals were slaughtered at 16 weeks postinfection, and the remaining six were slaughtered at 24 weeks. Disease was confirmed in all animals by the presence of gross pathology (visible lesions in the lungs and associated lymph nodes, as well as in the lymph nodes of the head region) typical for bovine TB and by the culture of *M. bovis* from tissue samples collected postmortem. Control sera (27 in total) were obtained from the same animals prior to challenge, as well as from 19 other uninfected animals fed the same diet and housed in an equivalent manner for up to 6 weeks.

All sera used for this study were stored frozen at −20°C until they were used for testing.

Sample preparation. The frozen serum samples were defrosted on ice to minimize the loss of volatile compounds. The serum samples (100 μl) were diluted (1:4) in 0.9% (wt/vol) NaCl solution and thoroughly mixed. The mixture was transferred into a 5-ml headspace vial (Macherey and Nagel, Loughborough, Leicestershire, United Kingdom) and immediately sealed with a crimp cap with a silicon-Teflon septum (Jaytee Bioscience Ltd., Whitstable, Kent, United Kingdom). The sealed headspace vials were subsequently incubated for 45 min at 37°C.

Gas-sensing system and headspace sampling. For this study, an electronic nose (model BH-114; Bloodhound Sensors, Leeds, United Kingdom) which employs 14 conducting polymers based on polyaniline was used. The sensor unit automatically set two calibration points. The first one was the baseline, which was obtained when activated carbon-filtered (carbon cap 150; Whatman) air was passed over the sensor at a flow rate of 4 ml min^{-1}. The second calibration point was a reference point obtained from the headspace of a control sample vial containing distilled water.

The interaction of the volatile compounds and the conducting polymer surface produced a change in electrical resistance, which was measured and subsequently displayed on a computer screen. Two sensor parameters were selected to study the sensor response: divergence (maximum step response) and area (area under the response curve). The sampling profile was set at 10 s of absorption and 15 s of desorption.

For the analysis of the headspace from test samples, the sample vials were connected to the electronic nose by inserting a needle into the headspace of the sample vials. The gas from the headspace was passed over the sensor surface at a flow rate of 200 ml min^{-1}, which was automatically set by the sensor unit. A time delay of 2 min was set between each measurement.

Data analysis. The multivariate data set was analyzed with an Excel add-in program (XLstat, version 3.4; XLstat, Paris, France). The data were analyzed by using principal component analysis (PCA) and linear discriminant function analysis (DFA). The identities of the serum samples (experiment, treatment group, and/or the time when the serum was collected) were disclosed to enable the PCA and DFA models to be constructed. However, in order to cross-validate the DFA model, a proportion of serum samples was withheld from building the model. The data from each withheld sample were then inserted into the discriminant functions of the completed model, and each withheld sample was subsequently assigned to the class for which its centroid has the smallest Euclidean distance to the unknown sample (38). This method provided a test of the accuracy of the DFA model.

(i) Principal component analysis. PCA is an unsupervised data reduction procedure. The procedure aims to describe the variation in a multivariate data set in terms of a set of uncorrelated variables, each of which is a particular linear combination of the original variables. In other words, the original data matrix is projected from a highly dimensional space into a less dimensional space, preferably of two or three dimensions. During the process, the original data set is reduced, i.e., compressed, with as little loss of information as possible. This outcome is achieved by filtering out the noise in the original data matrix without removing essential information described in the variance of the data (35, 38).

Mathematically, PCA aims to decompose the original $i \times j$ data matrix X into its $i \times k$ score matrix T, its $k \times j$ loading matrix P, and the residual matrix E according to the following formula: $X = TP + E$, where i is the number of samples, j is the number of variables, and k is the number of principal components (PCs). The PCs are determined on the basis of the maximum variance criterion. Each subsequent PC describes a maximum of variance which is not modeled by the previous one. According to this formula, the first PC contains most of the variance of the data (15, 38). The relationship between samples can be visualized by plotting the scores (PCs) against each other.

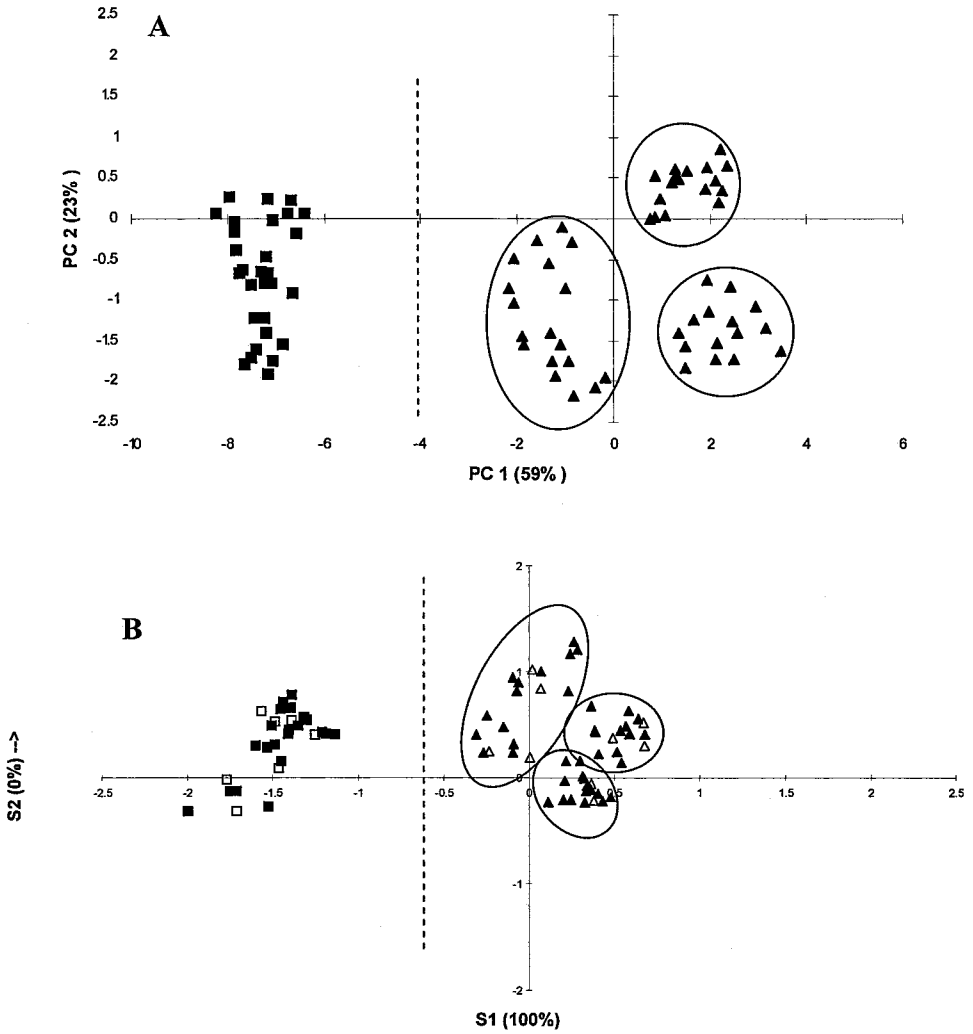

FIG. 1. PCA (A) and DFA (B) analyses of sera from badgers experimentally infected with three different doses of *M. bovis* (filled triangles) or from uninfected controls (filled squares). Blood was obtained from each animal 3 weeks prior to challenge, immediately prior to challenge, and at three weekly intervals after challenge for 15 weeks. For cross-validation, 15 samples were withheld from the building of the DFA model but were subsequently assigned correctly once the model was built (open squares, control sera; open triangles, infected sera). PC 1, principal component 1; PC 2, principal component 2; S1, discriminant function 1; S2, discriminant function 2. Numbers in parentheses indicate the percentages of the data matrix described by the relevant components and functions. The dashed lines were added by the authors.

(ii) Discriminant function analysis. Discriminant function analysis is a supervised classification procedure aimed at formalizing a decision boundary between different classes (38). The decision boundary is calculated so that the variance between different classes is maximized and the variance within individual classes is minimized (38). Different ways to calculate the decision boundary exist. In a multivariate data set, this action is done by solving an eigenvalue problem. The eigenvector (w) with the greatest eigenvalue (λ) provides the first discriminant function (s_1). The second discriminant function (s_2) is calculated from the eigenvector with the second-greatest eigenvalue. This procedure is continued until all discriminant functions are found to solve the discrimination problem (38). The original data set is visualized by plotting the individual discriminant functions against each other.

RESULTS

Analysis of sera from badgers infected with three different doses of *M. bovis*. Sera from experimentally infected and control badgers were used to determine whether the EN could discriminate infected animals from uninfected animals over the course of infection. Figure 1 shows the PCA and DFA analyses of the raw data obtained with the EN. It was possible to discriminate between control (uninfected) and infected samples by using the first two principal components (PC1 and PC2), which accounted for 82% of the variance (information) of the original data matrix. Furthermore, the sera from the infected badgers could be grouped into three further clusters coincident with the dose of *M. bovis* used to establish the infection (Fig. 1A), irrespective of the time between infection and collection of the serum sample. Based on the principal components, a discrimination model was built. As can be seen in Fig. 1B, only the first two discriminant functions were necessary to solve the discrimination problem. The DFA model was validated by the analysis of 15 withheld ("unknown") samples. All unknown samples were classified correctly as being either control or infected. Furthermore, all unknown infected samples were correctly assigned to one of the three subclusters according to the infection dose.

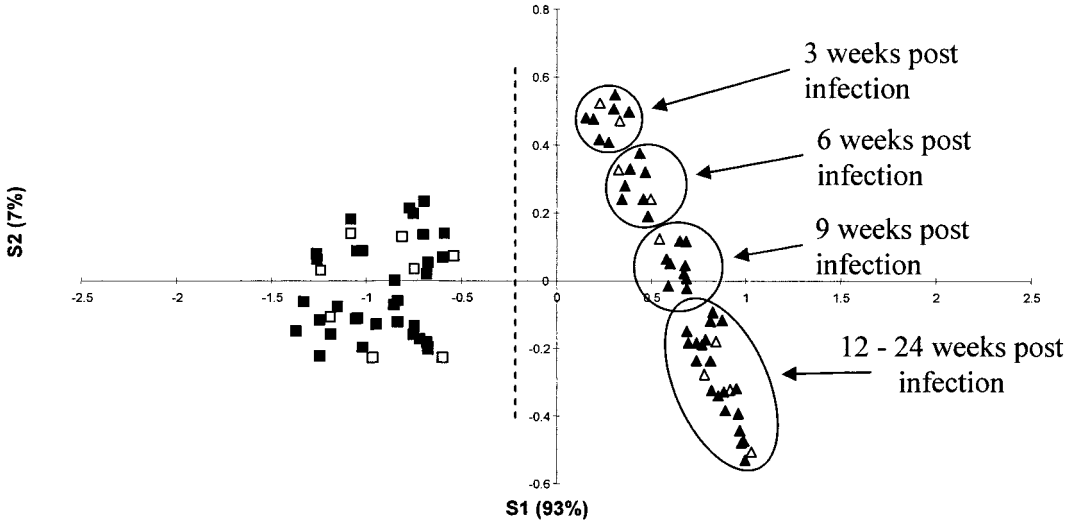

FIG. 2. DFA analysis of badger sera obtained at three weekly intervals after experimental infection with 10,000 CFU of *M. bovis* (filled triangles) for 24 weeks. Uninfected control sera (filled squares) were obtained at the same time points from a group of uninfected badgers and include sera from all animals, taken 3 weeks prior to challenge and immediately prior to challenge. For cross-validation, 17 samples were withheld from the building of the DFA model but were subsequently assigned correctly once the model was built (open squares, control sera; open triangles, infected sera). S1, discriminant function 1; S2, discriminant function 2. Numbers in parentheses indicate the percentages of the data matrix described by the relevant functions. The dashed line was added by the authors.

Analysis of sera from badgers over a time course of *M. bovis* infection. Next, the EN was used to analyze sera that were from badgers experimentally infected with a single dose of *M. bovis* but obtained over the course of infection. The raw EN data were analyzed by PCA (data not shown) and DFA (Fig. 2). As for the previous experiment, it was possible to discriminate between samples obtained from uninfected and infected badgers. Within the infected group, four subclusters could be identified. These four subclusters represented different time points after infection, namely, 3, 6, 9, and 12 to 24 weeks postinfection (Fig. 2). The discrimination model was validated against 17 unknown samples, all of which were correctly classified as being from either control or infected animals. Furthermore, all unknown infected samples were correctly assigned to one of the four subclusters according to the time after infection. It was not possible to distinguish between samples taken 12 or more weeks after infection.

Analysis of naturally infected badgers. In case the results obtained from experimentally infected badgers were peculiar to the means of infection, sera were tested from badgers naturally infected with *M. bovis*. The raw EN data were analyzed by PCA (data not shown) and DFA (Fig. 3). As for the experimentally infected badgers, it was possible to discriminate between infected and uninfected badgers. As might be expected from a more varied sample of animals, the variations within the two groups were bigger than that seen with the experimentally infected badger samples. The DFA model was validated by cross-validation with six samples. All six unknown samples (three from each group) were correctly classified as either uninfected or infected.

Infected calves. Based on the results obtained with badger sera, we extended the investigation to cattle sera. Application of the EN allowed the discrimination of infected cattle from control (uninfected) animals as early as 3 weeks after infection (Fig. 4). Four subclusters were identified within the infected cluster, representing different time points over the course of infection. Sera obtained 15 or more weeks after infection clustered together (Fig. 4). The DFA model was validated by cross-validation with 16 samples. All 16 unknown samples (half from the control group) were correctly classified as either uninfected or infected. Furthermore, the eight unknown infected samples were correctly assigned to one of the four subclusters according to the time after infection.

DISCUSSION

In 1923, Omelianski was one of the first to study and review the odor naturally liberated by microorganisms, among them *M. tuberculosis* (37). Since then, a number of techniques have been used to formally characterize the odors associated with pathogenic microorganisms or disease (reviewed in references 41 and 54). Using an EN to measure the volatile compounds produced from plate cultures, Gibson et al. classified 12 different bacteria and 1 pathogenic yeast with 93.4% accuracy (19). Recently, Pavlou et al. have demonstrated proof of principle that an EN can be used to detect volatiles in *M. tuberculosis*-infected human sputum liberated by 2 to 6 h of enzymatic lipase treatment (40, 41). We have extended these observations by using untreated serum and demonstrate that an EN can be used to differentiate badgers and cows with TB from uninfected animals. Strikingly, infections in badgers and cattle could be diagnosed as early as 3 weeks after experimental infection, which was the earliest time point examined.

PCA and DFA are two classical multivariate methods used to analyze EN data. The DFA model describes the probability that an unknown sample belongs to the class of closest similarity. For most unknown samples in this study, this probability was between 0.65 and 0.70. For the routine diagnosis of TB in

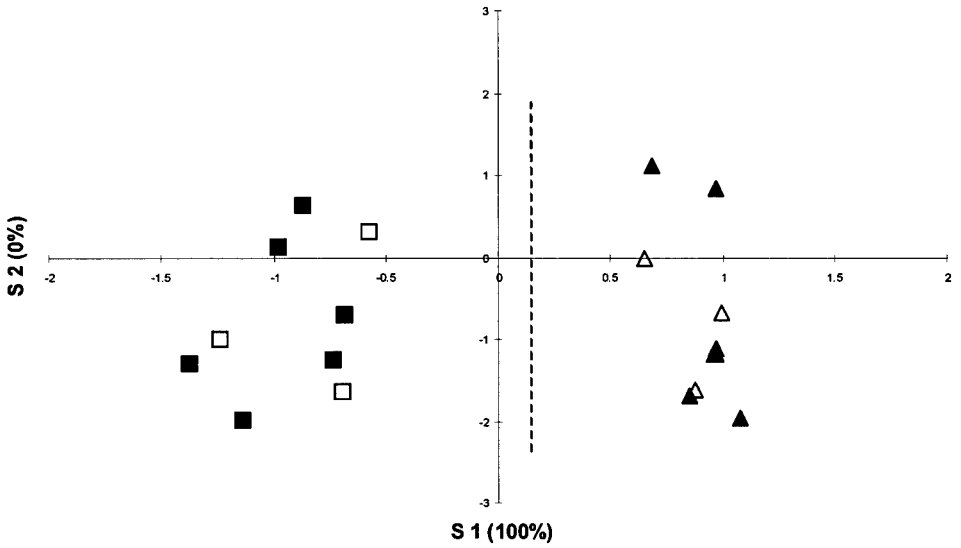

FIG. 3. DFA analysis of sera from wild badgers with culture-confirmed TB (filled triangles) or badgers TB negative by culture and postmortem analysis (filled squares). Blood was obtained from each animal antemortem. For cross-validation, six samples were withheld from the building of the DFA model but were subsequently assigned correctly once the model was built (open squares, control sera; open triangles, infected sera). S1, discriminant function 1; S2, discriminant function 2. Numbers in parentheses indicate the percentages of the data matrix described by the relevant functions. The dashed line was added by the authors.

cattle and badgers, it would be preferable to build a DFA model on a larger sample size, thereby giving a higher certainty of infection status, although it is likely that more appropriate analytical methods (such as neural networks) would be adopted at that stage. Notwithstanding the smaller sample size used in this study, the DFA model still classified all unknown samples (38 from badgers and 16 from cattle) correctly. At present, we do not know if the EN would discriminate *M. bovis* infection from other diseases that may be present in the individual, but it is likely that the naturally infected badgers would have harbored other infectious organisms, such as leptospires, coccidia, or enterococci (24, 33, 36). Nearly all the experimentally infected badgers, as well as the unchallenged badgers used in this study, had lungworm infection with associated pathology, which did not confound the analysis. However, the EN should be evaluated in situations of known infection, especially in the case of cattle infection with *Mycobacterium avium* subsp. *paratuberculosis* and other infections causing appreciable lung and other tissue pathologies, such as *Mycoplasma bovis*, *Haemophilus somnus* (*Histophilus somni*), and *Pasteurella* (*Mannheimia*) *haemolytica* (22, 51). It will also be important to establish whether BCG vaccination will influence the outcome of EN testing in either cattle or badgers.

The PCA analyses indicated that there was enough informa-

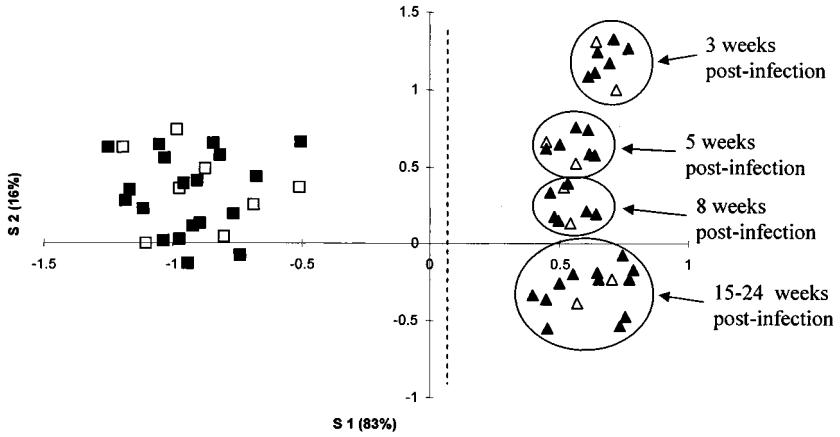

FIG. 4. DFA analysis of cattle sera obtained at the times indicated after experimental infection with *M. bovis* (filled triangles). Uninfected control sera (filled squares) were obtained from the same animals prior to challenge and include sera from other uninfected animals fed the same diet and housed in an equivalent manner for up to 6 weeks. For cross-validation, eight samples were withheld from the building of the DFA model but were subsequently assigned correctly once the model was built (open squares, control sera; open triangles, infected sera). S1, discriminant function 1; S2, discriminant function 2. Numbers in parentheses indicates the percentages of the data matrix described by the relevant functions. The dashed line was added by the authors.

tion present in the samples to allow discrimination between the infectious dose and time after infection, as well as between infected animals and controls. The EN could therefore be detecting increasing concentrations of volatile components resulting from increased bacterial loads and/or the host response to infection. We have consistently been unable to culture viable *M. bovis* from the blood of experimentally infected cattle or badgers, so it is unlikely the EN is detecting the presence of the bacterium itself. However, the observation that mycobacterial antigens can be detected in the serum of humans infected with *M. tuberculosis* and wildlife infected with *M. bovis* (2, 4, 34, 48, 50) suggests the EN may be detecting compounds released from the bacteria into the circulation during infection, e.g., fatty acids, esters, or lactones (16). Candidates for volatile host components could include molecules involved in the immune response or those that increase in concentration with increasing tissue pathology. There are numerous candidates for such components, including superoxide dismutase (5), mucins (26), and hepatocyte growth factor/scatter factor (31). At this stage, it is not possible to determine precisely which volatiles are responsible for the sensor response.

With the urgent need to develop more sensitive, rapid, and cost-effective means of diagnosing *M. bovis* infection in cattle and badgers, the EN approach described here offers considerable potential. The method is not only easy to perform, and therefore does not require a specifically trained technician, but is also cost- and time-effective, since, once validated, it would dispense with the need for the isolation of *M. bovis* by culture (which is protracted and costly) or repeated visits to the farm (in the case of the cattle skin test). Furthermore, the technology is amenable to automation and/or condensation into a portable device that could eventually permit the rapid testing of large numbers of animals in situ (14).

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