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Identification of *Salmonella Enterica* Serovar Enteritidis Vaccine Strains by Fourier Transform Infrared Spectroscopy

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Abstract

In cases of salmonellosis in humans, the most frequently isolated serovars of *Salmonella enterica* are Enteritidis (serogroup D1) and Typhimurium (serogroup B). In order to control the main sources of this zoonotic pathogen, poultry populations are treated with attenuated live vaccines. The distinction between vaccine strains and pathogenic wild type strains requires specific assays. For differentiation of two poultry vaccine serogroup D1 strains, TAD *Salmonella* vac® E and Salmovac SE, from other salmonellae of serogroup D1, a new method was developed by means of Fourier transform infrared (FT-IR) spectroscopy and artificial neural networks (ANN). To train the ANN, 548 spectra of 74 serogroup D1 isolates were used. Both vaccine strains could be differentiated from wild type strains of the same serogroup with an error rate of less than 1 % in replicate analyses. Moreover, this study revealed the potential of FT-IR spectroscopy for identifying salmonella strains to trace contamination routes.

Introduction

The *Salmonella* (*S.*) serovars Enteritidis and Typhimurium are the two most frequent serovars causing salmonellosis in humans in Germany. In this country a considerable source of these pathogens are poultry populations. 29 % of larger (1000+ animals) laying hen holdings were infected with *Salmonella* in the years 2004 to 2005 (EFSA, 2007). European efforts have striven to reduce the prevalence of these *Salmonella* types on affected farms as required by regulation, which recommends precautionary vaccination of the animals as a possible measure (regulation EG 2160/2003).

Immunization of poultry against *S.* Enteritidis infections is achieved by administering live vaccines, i.e. attenuated *S.* Enteritidis strains. These particular strains are not pathogenic to humans and, because of their auxotrophic metabolism, do not survive in the environment for long. After oral administration via drinking water, immunity develops within two weeks and persists for at least one year (Hahn, 1999). During the process of developing immunity, the animals excrete the vaccine strain at a declining rate (Hahn, 1999). In the case of positive findings of *S.* Enteritidis a clear distinction between the vaccine and potentially pathogenic wild type strains is crucial during subsequent examinations of this immunized livestock. The reason is that the finding of *Salmonella* can lead to considerable financial loss, if the eradication of the livestock and further disinfection should be necessary. Therefore, a rapid clarification of the result and a highly reliable and accurate analytical method are paramount.

For this purpose, there are special test kits available by vaccine manufacturers, such as AviPro® PLATE (Lohmann Animal Health GmbH & Co. KG, Cuxhaven) or the IDT *Salmonella* Diagnostikum (IDT Biologika GmbH, Dessau-Roßlau), which require an incubation of the sample strain in two media for 48 h. Differentiation by these kits is based on either the special nutrient requirements of the vaccine strains (IDT *Salmonella* Diagnostikum product documentation, version 01/2007) or on the sensibility to certain antibiotics (AviPro® PLATE product documentation version 7.0, 03/2012).

Since relying on only one physiological marker such as auxotrophy or an antibiotic resistance pattern alone may be risky in certain cases, it would

be useful to have an additional method available that is based on a different principle of analysis. A genetic approach such as a specific PCR could be such an alternative; however, it doesn't seem to be economically feasible.

In the past, Fourier transform infrared (FT-IR) spectroscopy was successfully applied for differentiation of gram-positive and gram-negative bacteria below the species level (Wenning et al., 2013; Kuhm et al., 2009; Rebuffo-Scheer et al., 2007). In case of *Salmonella* this quick and economic method was used to investigate phenotypic changes due to stress response (Alvarez-Ordóñez et al., 2010), for phage type identification (Preisner et al., 2010), and for serovar differentiation (Baldauf et al., 2006; Preisner et al., 2011).

In this study, FT-IR spectroscopy was evaluated for a reliable discrimination of *S.* Enteritidis vaccine strains and other *Salmonella* of serogroup D1.

A method for differentiation of the most prevalent *Salmonella* serogroups had already been established at CVUA Stuttgart (Pantchev et al., 2008). Based on this method, two submodules for finding vaccine strains amongst *S. enterica* of serogroup D1 were developed. Application of these two modules allows for differentiation between both the accredited vaccine strains TAD *Salmonella* vac® E (AviPro®, Lohmann Animal Health GmbH & Co. KG, Cuxhaven) and Salmovac SE (IDT Biologika GmbH, Dessau-Roßlau), respectively.

Materials and Methods

Strains

For this study, 74 previously serotyped salmonella strains were analyzed, belonging to the serovars Enteritidis (68 strains), Eastbourne (2 strains), and Lome, Sendai, Typhi and Gallinarum (one strain each). The vaccine strains Salmovac SE and TAD *Salmonella* vac® E belong to serovar Enteritidis and were isolated directly from the matrix of the commercial vaccine product. Three additional Salmovac SE isolates were obtained from poultry farm floors that are routinely probed by official veterinarians. All strains were isolated at CVUA Stuttgart, either from food or veterinary di-

agnostic samples. According to the White-Kauffmann-Le Minor scheme, all of them were classified into serogroup D1 (i.e. O:9) (Grimont & Weill, 2007). Serotyping was performed by slide agglutination with polyspecific anti-salmonella sera (groups A-E) followed by serogroup-specific, O-specific and H-specific sera (Sifin, Berlin).

Standard test for vaccine strain

The identification of Salmovac SE strains was performed using the *IDT Salmonella Diagnostikum* according to the manufacturers' instructions (IDT Biologika GmbH).

FT-IR spectroscopy

Strains were streaked out on sheep blood agar (Oxoid GmbH, Wesel) and incubated at 37 °C for 24 h (+/- 0,5 h). Cells were taken off the agar surface by means of a loop and suspended in 80 µl distilled water. 25 µl were transferred onto a zinc selenide (ZnSe) 96-well format optical plate (sample holder) and dried. For spectra recording, a FT-IR spectrometer TENSOR 27 combined with the HTS-XT Module used for microtiter plate scanning was employed. For data processing, the manufacturer's software OPUS 6.5 was used. Each isolate was analyzed in the course of at least six preparations; the five vaccine strain isolates a minimum of 24 times. Data preprocessing was performed using NeuroDeveloper software (Synthon GmbH, Heidelberg; Udelhoven et al., 2003). In case of Salmovac SE, the second derivative of the spectra within wave number range 550 cm^{-1} to 1200 cm^{-1} was calculated and subsequently vector-normalized. The resulting 674 data points were filtered by the NeuroDeveloper COVAR algorithm with respect to relevance of the target discrimination criteria. By help of a covariant matrix, this algorithm determines independent data points which give a highly significant difference between the two classes – in this case, vaccine strain vs. non-vaccine strain. These data points (n = 363) were used as input for the so-called input neurons for the artificial neural network (ANN).

To distinguish vac® E from its vicinity, in contrast to the above-mentioned procedures, a wave number range between 550 cm^{-1} and 1800 cm^{-1} was used, resulting in 1296 data points. Instead of using the COVAR algorithm, by means of a principle component analysis (PCA), 20 principle components were extracted and subsequently normalized via the autoscaling function.

Method development and cross validation

In order to assess the quality of differentiation, a cross validation was employed, by which all spectra were either used for the ANN training or for subsequent evaluation at different times.

Thus, spectra were first divided into three sets of 182, 183 and 183 spectra each. Division was carried out using stratified random sampling by strain. In each of the three training rounds, two sets served as a training basis for enabling the ANN to discriminate between the vaccine strains and field strains. Subsequently, the resulting net was validated using the remaining third set. The entirety of all three validations served to assess the quality of a net which was trained using spectra of all three sets simultaneously (Figure 1). In subsequent analyses, cross validation was automated using RapidMiner 5 software (Rapid-I GmbH, Dortmund); and data preprocessing (derivation, vector normalization) was performed using first OPUS and eventually RapidMiner 5 (wave number range restriction and principle component analysis). Three-dimensional data visualization was performed using POV-Ray 3.6 (Persistence of Vision Raytracer Pty. Ltd., Victoria, Australia).

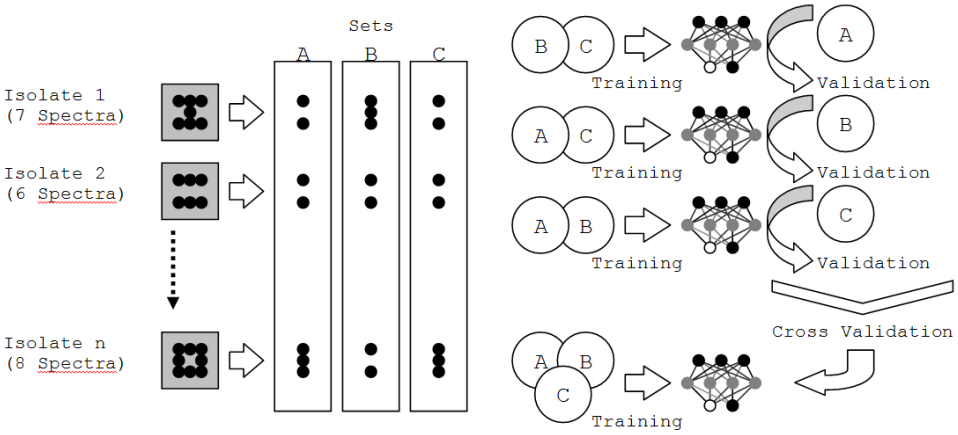

FIGURE 1. Stratified set formation and cross validation. Spectra of each isolate were evenly divided into three groups. Subsequent cross validation was performed in three rounds. In each round, two groups of spectra were used for training of an artificial neural network (ANN) with the object of being able to differentiate vaccine from non-vaccine strains. The outcome of this learning process was examined using the third group (validation). The combined validation result of all three rounds yielded the so-called cross validation. This served to assess the quality of an ANN which was subsequently trained with all spectra simultaneously.

Results

548 infrared spectra from 74 salmonella isolates were recorded. The capability of the vaccine strains Salmovac SE and TAD *Salmonella* vac® E to be differentiated from their vicinity was assessed by means of a cross validation. Results are shown in Table 1. 92 % of the Salmovac and 88 % of the VAC E spectra were correctly classified. Approximately 2 % of the Salmovac SE and 1 % of the vac® E spectra were incorrectly classified as non-vaccine strains. The ANN was not able to classify 6 % or 8 % of the spectra, respectively. No confusion between the two vaccine strains was noted. In contrast, spectra of non-vaccine strains were incorrectly classified as either vaccine strains or rendered doubtful in only less than 1 % of the cases.

TABLE 1. Confusion matrices of two cross validations. 548 spectra (100 Salmovac SE, 26 *Salmonella* VAC E, 422 from other *Salmonella* of serogroup D1) were classified by means of artificial neural networks (ANN). Sets of identical size were obtained by evenly dividing spectra of each isolate into three groups (stratification). Correct predictions are noted in bold; relative hit frequency is given in parentheses. A prediction noted as "uncertain" indicates that the ANN was not able to unequivocally allocate the respective spectrum.

Prediction \ Spectra from	Salmovac SE (4 Isolates)	**Non**-Salmovac SE (70 Isolates)
Salmovac SE	**92 (92 %)**	3 (1 %)
Non- Salmovac SE	2 (2 %)	**444 (99 %)**
Uncertain	6 (6 %)	1 (0.2 %)

Prediction \ Spectra from	VAC E (1 Isolate)	**Non**-VAC E (73 Isolates)
VAC E	**23 (88 %)**	1 (0.2 %)
Non-VAC E	1 (4 %)	**516 (99 %)**
Uncertain	2 (8 %)	5 (1 %)

As the next step, the data from the cross validation was used to calculate the probability of an isolate being classified as correct, doubtful or false, using a replicate analysis. This procedure corresponds to the routine examination and evaluation of sample-isolates. The average of these results was taken for all isolates; the vaccine strain Salmovac SE was classified correctly in 95.6 % of the cases, and falsely in 0.2 %. Similarly, TAD *Salmonella* vac® E was allocated correctly in 95.6 % of all cases and incorrectly for 0.2 %. The respective probabilities for non-vaccine strains are at least 98.7 % for a correct and a maximum of 0.1 % for a false classification.

For visualization of the separability of an isolate from its vicinity, the spectral data points of TAD *Salmonella* vac® E with reference to all other data points are displayed in a three-dimensional model (Figure 2). For the three

dimensions, three principle components were chosen which strongly discriminate VAC E values from the values of other isolates.

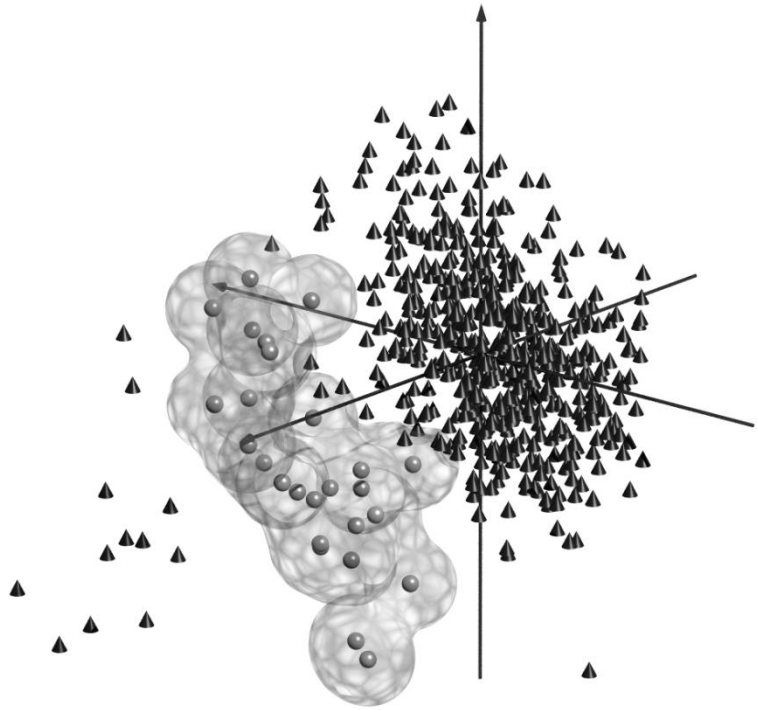

FIGURE 2. Three dimensional illustration of 548 spectral data points. TAD *Salmonella* vac® E data points are depicted as light grey balls, others as black cones. The three dimensions of the illustration correspond to three chosen components from a principal component analysis of all spectra. Using these three components, VAC E is clearly distinguishable from its vicinity. For further clarification of separability, the space around the data points is denoted by a virtual surface.

In order to investigate the separability of other isolates from their vicinity in a similar fashion, RapidMiner was used to train ANNs for the differentiation of all 74 isolates and subsequent cross validation. As a result, three isolates (one of which being vac® E) were correctly identified at a level of at least 90 % and a further six strains at a level of at least 70 %. In subsequent steps, isolates that were not separable were manually combined in joint classes. This procedure was repeated several times. Eventually, 20

well separable classes resulted in this way, none of which had a hit rate of less than 81 %. The mean hit rate was at a level of 93 % (Figure 3). As expected, the four Salmovac SE vaccine strain isolates clustered together as a class of their own. The TAD *Salmonella* vac® E vaccine strain formed its own class, which was also the case for a further seven isolates, these consisting of one of the two *S.* Eastbourne isolates, the serovar Lome and Typhi isolates and four of the Enteritidis serovars.

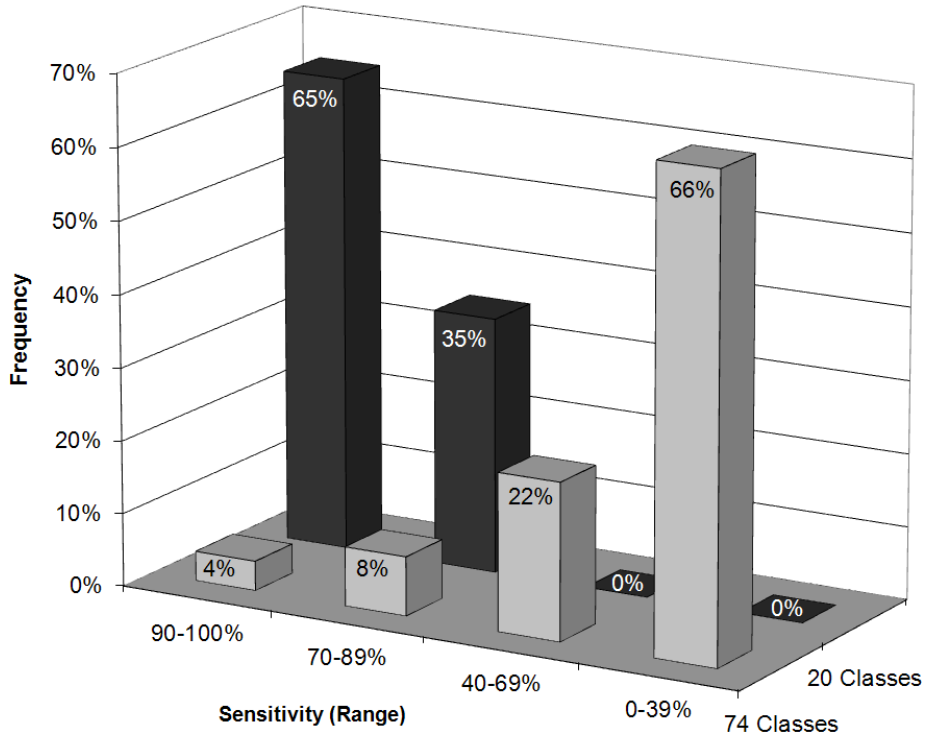

FIGURE 3. Hit rates of artificial neural networks for identification of all 74 individual isolates (light grey). Isolates that were not distinguishable in the confusion matrix were combined to create joint classes which, after several steps, amounted to 20 clearly distinguishable classes (dark grey, mean hit rate of 93 %). Values were derived from the arithmetic means of three separate cross validations with three groups. Calculations were performed using RapidMiner and Microsoft Excel.

Discussion

Different research groups have demonstrated the capability of FT-IR spectroscopy to distinguish between salmonella at a sub-species level. Several serotypes from different serogroups (Kim et al., 2005; Baldauf et al., 2006; De Lamo-Castellví et al., 2010; Preisner et al., 2011) as well as *S.* Enteritidis phage types (Preisner et al., 2010) have been successfully differentiated.

At CVUA Stuttgart several hierarchically stacked classification modules are used for differentiation of bacterial isolates by FT-IR spectroscopy (Kuhm et al., 2009; Pantchev et al., 2008; Rau et al., 2009). They are based on artificial neural networks and manage to classify the obtained spectra into the respective family, genus, species etc. group levels. The modules presented here follow an existing method previously developed at CVUA Stuttgart. By using this method, salmonella are differentiated from a background of other gram-negative bacteria and identified at the serogroup level (Pantchev et al., 2008). In accordance with their prevalence in food and veterinary samples, serogroup D1 isolates were available in greater numbers. Because of that, identification of D1-*Salmonella* was possible using extensive infrared spectroscopy data. As expected, the vaccine strains *Salmonella* VAC E and Salmovac SE were classified as D1-serogroup *Salmonella* when using the above mentioned modules.

The modules described here, which follow the previous serogroup module (Pantchev et al., 2008), discriminate between the strains TAD *Salmonella* vac® E and Salmovac SE within the D1 serogroup, represented by 74 isolates. While probabilities of 91.9 % and 95.6 % were obtained for correct allocation (i.e. sensitivity), respectively, the probability for a false result was less than 1 %. This shows that the isolates' infrared spectra carry significant information even for correct recognition of the attenuated vaccine strains.

An external validation with strains that were not included in this method setup would currently be possible only with *Salmonella* D1 field strains (non-vaccine strains). More specifically, further vaccine strain isolates from poultry farms which have been passaged within the animals are not available at this point in time. Investigating these would enable the as-

sessment of a potential phenotypic drift of the vaccine strains over the course of the infection.

While three Salmovac SE strains have been included in the database, further strains would be useful for an external validation. For vac® E, isolates from treated animals were not available.

As demonstrated for the vaccine strains, a differentiation of individual salmonella strains (the vaccine strains, in this case) is possible. Thus, the question arose as to whether FT-IR spectroscopy would also be capable of a direct comparison in an epidemiological context, e.g. between a human pathogenic isolate and an isolate from the food consumed. Therefore, the existing data set was analyzed in order to group the 74 *Salmonella* D1 isolates into classes.

As expected, it was not possible to assign each isolate to an individual class of its own. However, by successive combination of similar isolates, 20 classes, so called “spectrovars” were able to be defined within the present data set. These might enable contamination route trace analyses, similar to fine-typing using molecular biological methods. In reference laboratories, fine-typing is frequently done by pulsed-field electrophoresis (PFGE) or, specifically in the case of *Salmonella*, by MLVA (Multiple-locus variable-number tandem-repeat analysis) as multiplex-PCR (Cho et al., 2007). The latter displays a similar or even higher discrimination potential than PFGE (Lindstedt et al., 2003). This potential will, in all likelihood, not be reached with FT-IR spectroscopy. However, the spectroscopical method is impressive, both in terms of its speed and simplicity in handling as well as its flexibility in reacting to individual issues that may arise. In addition, FT-IR spectroscopy’s potential for fine-typing of strains has not been fully exhausted here, as sample preparation was intended to be as simple as that of performing species identification. In order to spectroscopically emulate classical phage typing methods used for *Salmonella* fine-typing, greater preparatory efforts must be undertaken. Other research groups have demonstrated phage type analysis to be possible by means of FT-IR spectroscopy, if outer membrane protein extracts are analyzed instead of whole bacterial cells (Preisner et al., 2010). An increase in differentiation capability between serotypes can be obtained by suspending bacterial cells in 50 % acetonitrile in the course of sample preparation (Baldauf et al., 2007).

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