Deliverable 4.6.

Assessment of the potential effect of the withdrawal of the use of formaldehyde-based feed treatments done.

JRP6 - NOVA - FBZ1 - 1st Call

Responsible Partner: UCM-VISAVET

GENERAL INFORMATION

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Deliverable 4.2.

Assessment of the potential effect of the withdrawal of the use of formaldehyde-based feed treatments done.

This deliverable was modified from its original objective (assessing the impact that the withdraw of formaldehyde-based feed treatments could have on the risk of *Salmonella* infections in farms) as reported in the 12 month report in 2020 due to the change in the legislation that forbid the use of this product (that occurred after the WP was designed) and that thus did not allow us to collect valid information to perform the assessment. For this reason, and building on other deliverables from this WP that are focused on the spatial epidemiology of the infection with Salmonella in the animal reservoir (with a focus on food animals), we have replaced the original objective of this deliverable to the **"Assessment of the usefulness of antimicrobial susceptibility test results for the optimization of surveillance programs for Salmonella of swine origin".**

The deliverable D.4.6. "Assessment of the usefulness of antimicrobial susceptibility test results for the optimization of surveillance programs for Salmonella of swine origin" is a subproduct of Task-4.1 and further helps to understand the spatio-temporal patterns of infection distribution in livestock with a focus on multi-drug resistant phenotypes to optimize sampling strategies.

1. Background

Salmonella is one of the most common public health problems, causing significant human morbidity and even mortality and consequently high economic losses in both developing and developed countries, and the emergence of antimicrobial resistance further aggravates this problem. Resistant non-Typhoidal *Salmonella* strains that show multidrug-resistant phenotypes, such as *S*. Typhimurium phage type DT104 (characterised by penta-resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline – ACSSuT), are especially of concern from a public health perspective (8). Many phenotypic resistant patterns (resistotypes) have been reported in *Salmonella,* with their distribution and prevalence changing over time and space. The interplay between *Salmonella* serotypes and resistotypes further complicates the picture, since the serotype of *Salmonella* has been shown to be associated with the pathogenicity, virulence, host range, and, most relevant here, AMR profile, and certain serotypes tend to show higher levels of resistance. For example, the proportion of MDR strains among *S*. Derby human clinical isolates in Europe in 2017 was lower than among *S*. Typhimurium and *S*. 1,4,[5],12:i:-. Serotype-specific evolutionary patterns of AMR have been also suggested for other serovars such as *S*. Dublin, and *S*. Newport (13).

Foods of animal origin are still one of the major sources of infection for the general public, with eggs, broiler chickens and pigs being consistently identified among the top attributed food sources. However, while control programmes for *Salmonella* in poultry have been applied in the whole UE with high success, only few European countries have implemented eradication or control programmes of

Salmonella in swine. In parallel, national monitoring programs on antimicrobial resistance is routinely conducted according to the European legislation so that up to 180 isolates from the different hosts (poultry, swine, cattle) are tested each year/every two years, but this information is typically analyzed only in an aggregated manner, and is not used to further optimize monitoring efforts aiming at the identification of strains/resistotypes posing a public health threat with a potentially higher impact.

2. Objectives

The objective of the deliverable D.4.6 was to assess the usefulness of antimicrobial susceptibility test results for the optimization of surveillance programs for *Salmonella* of swine origin. In order to achieve this objective, a set of univariate and multivariate analytical approaches were applied to a large dataset containing information on antimicrobial resistance in swine Salmonella strains building on a study conducted previously on poultry isolates. More specifically, the following methodological approaches were applied:

A. Spatial and spatiotemporal analysis: The proportion of resistant isolates to each of the 12 antimicrobials at the province level was calculated and later adjusted using empirical Bayesian smoothing at the province level. For the empirical Bayesian smoothing, the neighbouring relationships were described by Queen contiguity, considering two provinces as neighbours when they shared at least one point of their boundaries. Furthermore, generalized estimating equations (GEE) with a logit link function were used to evaluate the evolution of the proportion of resistant strains to each antimicrobial over time. An exhaustive search to obtain the best model describing the temporal trends for the antimicrobials, consisting on the inclusion of an interaction between an antimicrobial and the linear year term and if the interaction held, the interaction with the quadratic year term, was conducted.
B. Bayesian networks, graphical models where vertices represent random variables and arcs represent probabilistic dependencies between them, were built for binary AST results. Hill-Climbing greedy search was applied to identify directed network structure. Arcs were retained in the networks if their empirical frequency in 100,000 bootstrap samples was ≥40%. Four Bayesian networks were built on different subsets of data (see below)
C. Hierarchical clustering analysis was carried out for both binary logarithms of the MICs and binary AST results. Hierarchical trees were constructed using Ward's minimum variance method based on Euclidean distances, and the number of clusters was determined when the partition had the highest relative loss of inertia. To increase the chance of revealing potentially meaningful clusters that were missed by using the algorithm, a pre-set cluster number that was one more than what was determined by the algorithm was also applied. The composition of binary AST results of the antimicrobials and the serotype of the isolates were elicited for each cluster, and the percentage of isolates of a cluster belonging to each specific province was depicted on the map.

The data on *Salmonella* in pigs was obtained from the Spanish Veterinary Antimicrobial Resistance Surveillance Network database, covering the period 2001 to 2017. Eleven antimicrobials for which AST results were available during most of the study period were considered in the analysis and grouped in two datasets. The first dataset (D1) included the AST results to seven antimicrobials [tetracycline (TET), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), gentamicin (GEN), florfenicol (FFC), and

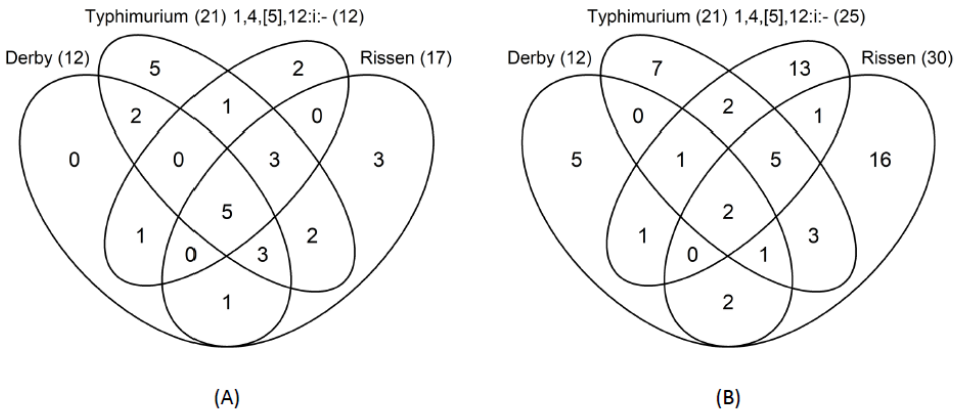
cefotaxime (CTX)] from 2001 to 2013. The second dataset (D2) contained the AST results to the aforementioned antimicrobials except for FFC, as well as to sulfamethoxazole (SMX), ampicillin (AMP), trimethoprim (TMP) and ceftazidime (CAZ), which were tested in isolates collected from 2008 to 2013 and 2017 (no sampling was conducted in swine in 2014 and 2016, and no results were available for 2015). The year isolates were recovered and their serotype was also included in the datasets.

3. Progress of the activities: main results.

A) Descriptive results and identification of spatial and temporal trends in AMR levels in *Salmonella* from swine.

A total of 1,318 pig Salmonella isolates belonging to 63 different serotypes and retrieved between 2001 and 2017 were included in at least one of the datasets. The yearly number of isolates ranged from 40 to 211. D1 consisted of 1,154 isolates from 58 serotypes, of which 23.5% were S. Typhimurium, 21.2% were S. Rissen, 16.6% were S. Derby, 10.1% were S. 1,4,[5],12:i:-, 5.2% were S. Anatum, and 4.7% were S. Bredeney. D2 included 680 isolates from 46 serotypes of which the most frequent were Rissen (23.9%), 1,4,[5],12:i:- (21.1%), Typhimurium (19.9%), Derby (14.1%), Anatum (2.5%) and Bredeney (2.5%).

Twenty-nine out of the 256 (2^7) possible resistotypes in D1 were observed, (21 in *S*. Typhimurium, 17 in *S*. Rissen, 12 in *S*. Derby, and 12 in *S.* 1,4,[5],12:i:- isolates) (Figure 1). Almost 15% (n=170) of the isolates were susceptible to all seven antimicrobials. Isolates with a MDR resistotype accounted for 8.9% (n=103) of all isolates, and the annual percentage of MDR isolates went from 30.1% (n=22) in 2001 to only 1.6 (n=1) in 2008. However, it then increased to 7.2–10.4% in 2011–2013. In D2, 65 resistotypes were observed among the 1024 (2^{10}) possible combinations of resistances. There were 21 resistotypes in *S*. Typhimurium isolates, 30 in *S*. Rissen isolates, 15 in *S*. Derby isolates, and 25 in *S*. 1,4,[5],12:i:- isolates (Figure 1), and more than half of the resistotypes of *S*. Rissen and *S*. 1,4,[5],12:i:- isolates were serotype-specific. Around 15% of the isolates (n=103) were susceptible to all the antimicrobials, which is similar to the proportion in D1. However, there was a higher percentage of MDR isolates (54.0%, n=366) due to the inclusion of two antimicrobials against which high levels of resistance (SMX and AMP), and an increasing trend in the annual proportion of MDR isolates was observed (from 37.5% in 2008 to 62.8% in 2017).



(A) (B)

Figure 1. Venn diagrams illustrating the number of resistotypes in *Salmonella* isolates of serotypes Typhimurium, Rissen, Derby, and 1,4,[5],12:i:- from pigs recovered through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme. (a) contains 1,154 isolates with seven antimicrobial susceptibility results between 2001 and 2013; (b) contains 680 isolates with ten antimicrobial susceptibility results between 2008 and 2017.

The empirically adjusted spatial distribution of the proportion of isolates resistant to each of the 12 antimicrobials is shown in Figure 2. The percentage of isolates resistant to TET across Spain was the highest among all antimicrobials and ranged from 66.7% in Toledo to 95.8% in Cadiz, followed by SMX (range: 42.5%−77.8%), STR (range: 45.7%−76.7%), AMP (range: 24.3%−66.7% with a lower percentage of resistance in the South-East of Spain), and CHL (range: 8.5%−41.1%). A higher proportion of isolates were resistant to TMP (range: 7.1%−58.3%) in the southwest of Spain. The percentage of resistant isolates for CIP (range: 4.3%−19.3%) and NAL (range: 3.1%−18%) was lower at the northwest corner of Spain; in contrast, the value for FFC (range: 0%−20.0%) was higher there.

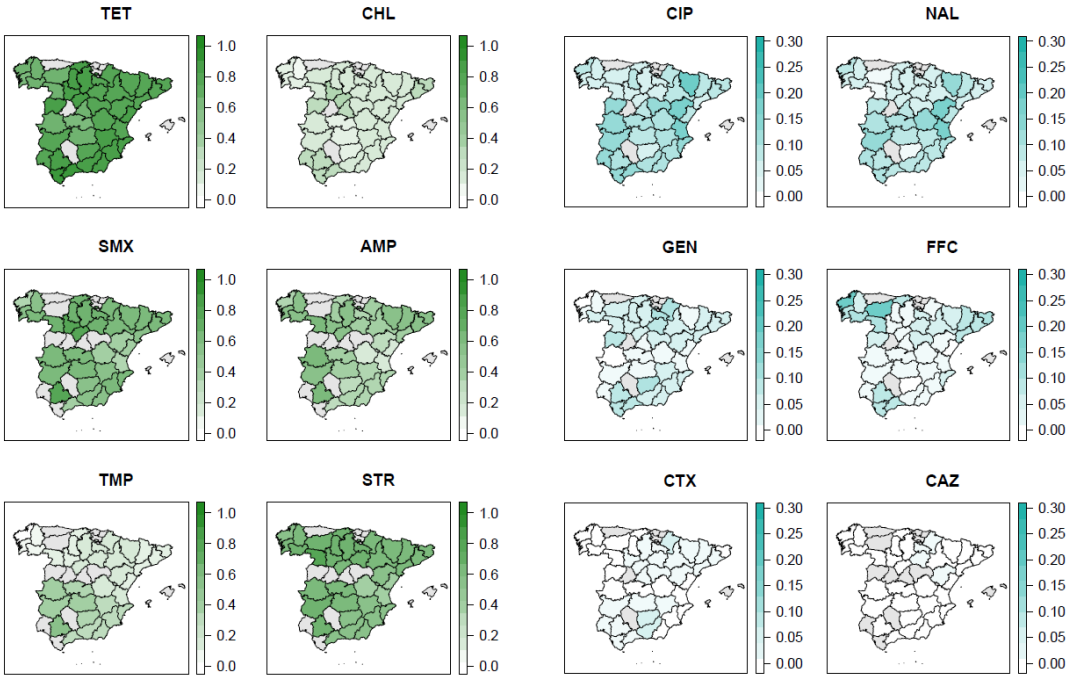


Figure 2. Spatial trends adjusted by empirical Bayesian smoothing in the proportion of *Salmonella* isolates from pigs resistant to specific antimicrobials, collected through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme towards twelve antimicrobials from 2001 to 2017.

Regarding temporal trends, a significant change in the percentage of resistant isolates over time during the 2001-2013 period was found for all the antimicrobials except NAL. While the change for CHL was linear, the quadratic year terms for TET, CIP, GEN, FFC and CTX were included in the final model (Figure 3). A much higher percentage of isolates resistant to TET (median: 82.7 and range: 63.6−97.8% among the study period) than to other antimicrobials (median values < 23.9%) was observed. During this period, the percentage of resistant isolates for TET, CHL and GEN first decreased and then increased again, and the odds of being resistant to CHL decreased by 8.9% [95% confidence interval (CI):

5.1−12.5%] each year. The percentage of CTX and FFC resistant isolates to have mostly remained between 0 and 10%.

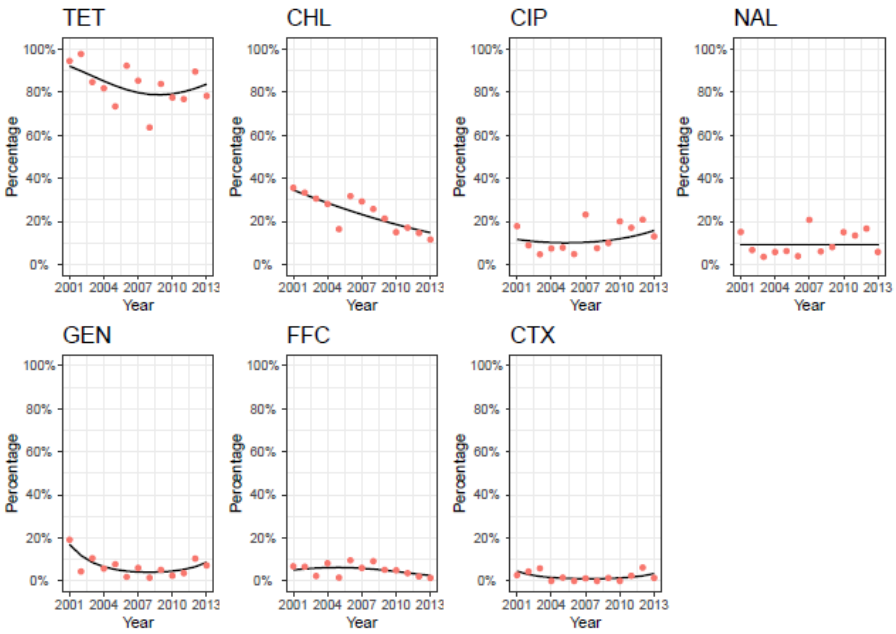


Figure 3. Temporal trends in the percentage of resistant *Salmonella* isolates from pigs collected through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme towards seven antimicrobials from 2001 to 2013. Red dots are the observed percentage of resistant isolates; black lines are fitted values of generalized estimating equation models using the binary results (i.e., resistant and susceptible).

If the data contained in D2 is considered, A relatively high level of resistance to TET, SMX, AMP (median values > 53.7%) compared to other antimicrobials (with median values < 27.1%) was observed. A significant change in the percentage of resistant isolates between 2008 to 2017 was found for all the antimicrobials except TET, NAL and CAZ (Figure 4). The percentage of isolates resistant to CIP, GEN, AMP increased over this period, and the odds of isolates being resistant to AMP was 1.12 times (95% CI: 1.08−1.17) higher than the previous year. Although the percentage of resistant isolates to CHL, SMX and TMP seemed to have decreased from 2008 to 2013, the values in 2017 increased significantly. This figure for CTX and CAZ have remained below 10% over the years.

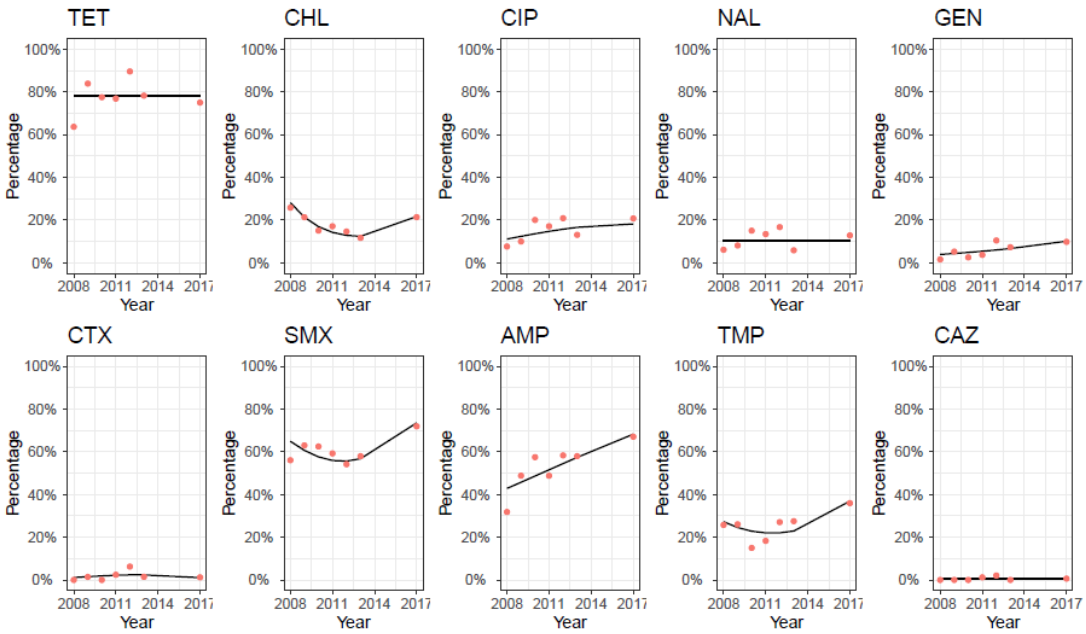


Figure 4. Temporal trends in the percentage of resistant *Salmonella* isolates from pigs collected through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme towards ten antimicrobials from 2008 to 2017. Red dots are the observed percentage of resistant isolates; black lines are fitted values of generalized estimating equation models using the binary results (i.e., resistant and susceptible).

The estimates of the pairwise correlations obtained from the GEE modelling using D2 are shown in Figures 5. As expected, the correlation coefficient for CIP and NAL was the highest among all pairs (0.82), and the correlation between CTX and TAZ was also relatively high (0.49). Among antimicrobials from different classes, SMX and AMP had a high correlation coefficient at 0.71, and both had relatively high correlation coefficients with TET, CHL and TMP (range: 0.33-0.51). GEN did not appear to be correlated with any other antimicrobials.

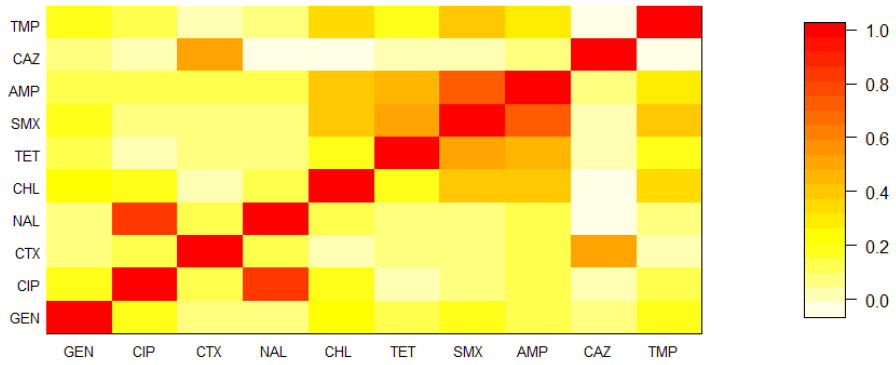


Figure 5. Estimated pairwise correlations obtained from the generalized estimating equations for the binary results (i.e., resistant and susceptible) of antimicrobial susceptibility testing for ten antimicrobials.

B) Bayesian network analysis

The overall Bayesian network was identified for D1 and D2 (Figure 6), although networks using data of different period for both D1 and D2 were also determined. For D1, five relations were built with CHL being located in a central position. Apart from the relation between GEN-TET, all the others were also maintained in at least one of the partial networks. Across these networks, all isolates apart from one that was susceptible to CIP were susceptible to NAL (n=1,021), but only 74.2% to 84.6% of isolates that were resistant to CIP (n=131) were also resistant to NAL. All the isolates that were resistant to FFC were the same for CHL, accounting for 22.1% of the 876 isolates that were resistant to CHL. SMX was located at the centre of the overall Bayesian network with 10 edges for D2.

Associations between CIP and NAL and between SMX and TET, CHL, AMP and TMP also persisted in all other partial networks, and those between CIP and GEN and between CAZ and CTX were found in the overall and the 2011-2017 networks. Although all the D2 isolates susceptible to SMX were the same for CHL, only 31.0% resistant to SMX were also resistant to CHL. Most of the isolates resistant to TMP were the same for SMX, accounting for 41.3% of the SMX-resistant isolates. Furthermore, all the isolates resistant to CAZ were resistant to CTX. Although networks of D1 suggested associations of the resistant pattern of CHL with the patterns of both TET and GEN, in the networks using D2 that had more AST results (but fewer isolates), the resistant pattern for CHL became independent from the one for TET and more dependent on the patterns for SMX and TMP (not included in D1).

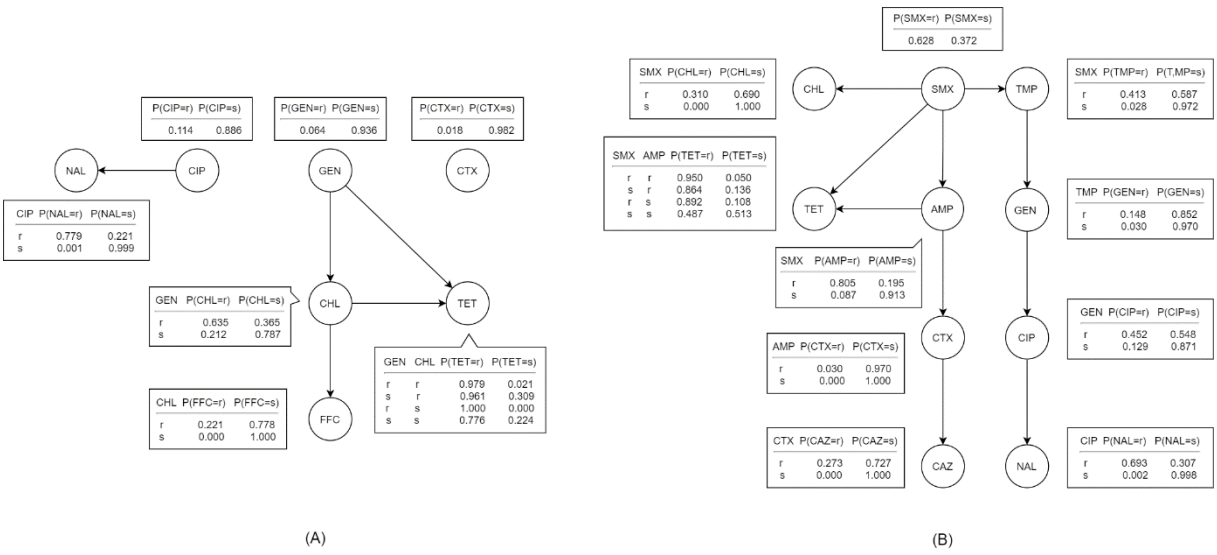


Figure 6. Bayesian networks for (A) seven and (B) ten binary antimicrobial susceptibility testing results of *Salmonella* isolates from pigs collected through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme between 2001 and 2013 for (A) and between 2008 and 2017 for (B). TET: tetracycline; CHL: chloramphenicol; CIP: ciprofloxacin; NAL: nalidixic acid; GEN: gentamicin; FFC: florfenicol; CTX: cefotaxime; SMX: sulfamethoxazole; AMP: ampicillin; TMP: trimethoprim; CAZ: ceftazidime; r: resistant; s: susceptible.

C) Hierarchical clustering analysis

For D1, three and six clusters were generated using binary logarithms of the MICs and binary AST information, respectively, while the number of clusters for D2 was three and four. When considering only the results of the binary AST results (resistant/non-resistant), for D1

Six clusters were identified for D1. Cluster 1 included 185 isolates (16%) which were susceptible to almost all seven antimicrobials (with a few resistant to either CHL and/or CIP) (Figure 7). The most abundant serotype in this cluster was Bredeney (36 isolates, accounting for two-thirds of all *S*. Bredeney isolates in D1), and several other serotypes were also overrepresented (>75% of all isolates from serotypes Montevideo, Enteritidis and Infantis). In contrast, the most prevalent serotypes in the D1 collection, including Rissen, *S*. Derby, S. Typhimurium and especially *S*. 1,4,[5],12:i:- were under-presented in Cluster 1 (with only between 2 and 22 isolates from each). Cluster 2 was the largest (n=720, 62.4%) and characterised by resistance to TET and susceptibility to all other antimicrobials except CHL (17.6% resistance). Serotype distribution in Cluster 2 was similar to that observed in the whole D1 panel with *S*. Rissen (27.8%; n=200) and *S*. Derby (20.3%; n=146) being overrepresented and *S*. Bredeney being underrepresented (1.5%, n=11).

Resistance to both TET and GEN was the predominant feature present in all isolates in Cluster 3 (n=58, 5.0%), with close to 60% of them being also resistant to CHL and susceptible to the remaining antimicrobials. The most common serotypes were *S*. 1,4,[5],12:i:- (29.8%; n=19) and Brandenburg (22.8%; n=13, representing 42% of all isolates from this serotype in D1). Close to all isolates in Cluster 4 (n=61, 5.3%) were resistant to TET, CHL and FFC and susceptible to CTX, and 85−92% isolates were susceptible to CIP, NAL and GEN. This cluster is dominated by *S*. Typhimurium (86.9%; n=53) with three (4.9%) *S*. 1,4,[5],12:i:- isolates. Isolates in Cluster 5 (n=21, 1.8%) were resistant to TET and CTX and susceptible to FFC. *S*. Typhimurium accounted for 57.1% (n=12) of the isolates; 14.3% (n=3) were *S*. Anatum and *S*. Rissen, respectively, and 9.5% were *S*. Derby (n=2). Cluster 6 consisted of 109 isolates (9.4%), including isolates that were typically resistant to CIP, NAL and TET and susceptible to GEN, FFC and CTX. *S*. Wien (9.2%; n=10),

S. Kapemba (8.3%; n=9), *S*.Brikama (4.6%; n=5) were overrepresented in Cluster 6, and the only isolates of *S*. Essen (n=3) and *S*. Choleraesuis (n=2) in D1 were in this cluster.

Figure 7. The proportion of 1,154 *Salmonella* isolates from pigs collected through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme between 2001 and 2013 resistant to seven antimicrobials (left) and the composition of serotypes in each of the clusters determined (right) by hierarchical clustering using binary antimicrobial susceptibility testing results. Cluster 0 represents all isolates in panel D1. Only serotypes accounting for ≥5% of the isolates in each particular cluster are shown in the graph.

The spatial distribution of the proportion of isolates belonging to each of the clusters is shown in Figure 8. While no obvious spatial patterns were observed for Clusters 1 and 2, a higher proportion of isolates of Cluster 3 was seen in the North and South of Spain. Northern provinces and provinces adjacent to the Mediterranean Sea had a larger proportion of isolates of Cluster 4. Some provinces in the Northeast and the South harboured more isolates belonging to Cluster 5. Last, the proportion of isolates of Cluster 6 seemed lower in the West of Spain, except for province Cáceres.

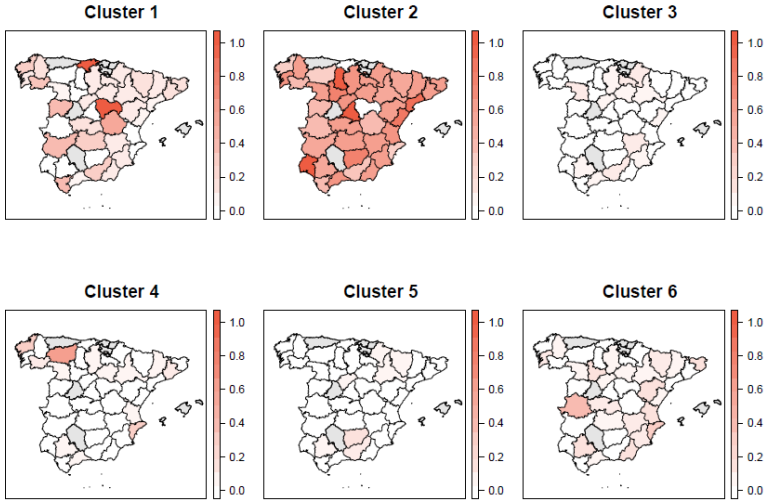


Figure 8. Spatial distribution of six clusters elicited from hierarchical clustering using binary antimicrobial susceptibility testing results of 1,154 *Salmonella* isolates from pigs collected through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme between 2001 and 2013.

When the information contained in D2 is considered, four clusters with 227 (33.4%), 366 (53.8%), 84 (12.4%) and 3 (0.4%) isolates, respectively, were identified using the binary AST results in D2. In Cluster 1, half of the isolates were resistant to TET, and most were susceptible to the rest of the antimicrobials. Compared to the percentage of isolates of different serotypes in the whole D2, Cluster 1 had higher percentage of *S*. Rissen (30.8%; n=70) and *S*. Derby (20.3%; n=46) and much lower percentage of Typhimurium (6.6%; n=15) and *S*. 1,4,[5],12:i:- (4.8%; n=11) (Figure 9). All the S. Montevideo (n=7) and *S*. Infantis (n=5) isolates and 3 out of the 5 *S*. Enteritidis isolates in D2 belonged to this cluster. Most of the isolates in Cluster 2 were resistant to AMP, SMX and TET, and susceptible to CIP, NAL, GEN, CTX, and CAZ. This cluster was dominated by *S*. 1,4,[5],12:i:- (n=122; 33.3%) and *S*. Typhimurium (n=96; 26.2%), and more than 90% of the *S*. Wien isolates (n=10) were in this cluster. Cluster 3 was different from Cluster 2 in the addition of very high (>84.5) levels of resistance to CIP and NAL. The percentage of *S*. Rissen, *S*. Derby and *S*. Typhimurium were similar to the one of Cluster 2, but *S*. 1,4,[5],12:i:- only accounted for 11.9% (n=10). All the *S*. Kapemba isolates (n=6) in D2 belonged to this cluster. Finally,

Cluster 4, which included only three isolates (all from different serotypes), was mainly characterised by full resistance to CAZ.

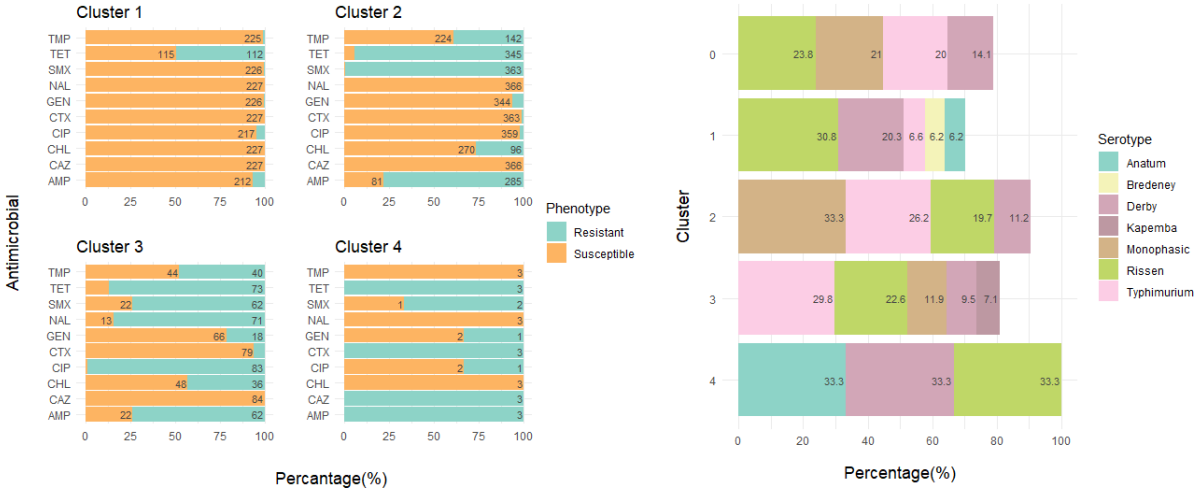


Figure 9. The proportion of 680 *Salmonella* isolates from pigs collected through the Spanish Veterinary Antimicrobial Resistance Surveillance Network programme between 2011 and 2017 resistant to seven antimicrobials (left) and the composition of serotypes in each of the clusters determined (right) by hierarchical clustering using binary antimicrobial susceptibility testing results. Cluster 0 contained all isolates. Only serotypes accounting for ≥5% of the isolates in each particular cluster are shown in the graph.

Figure 10 shows the proportion of isolates originating from different provinces in Spain in different clusters. Although there were no isolates from many of the provinces in the West, some patterns might be observed. A higher proportion of isolates in Cluster 1 was in the East than the West, which seemed to be the opposite for Cluster 2. The proportion of isolates in Cluster 3 was higher in the far East and the far West of Spain, and all the isolates in Cluster 4 were in province Zaragoza.

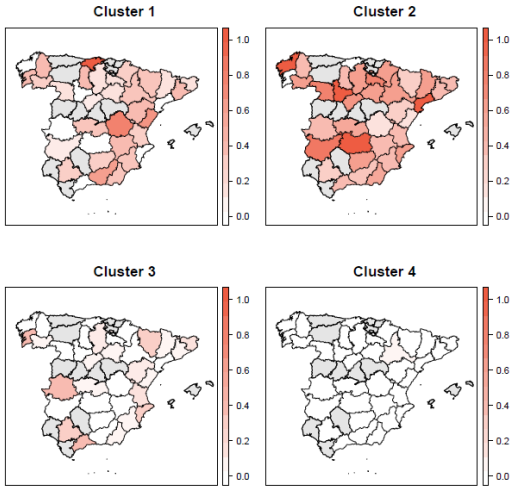


Figure 10. Spatial distribution of six clusters elicited from hierarchical clustering using binary antimicrobial susceptibility testing results of 680 *Salmonella* isolates from pigs collected through the

Spanish Veterinary Antimicrobial Resistance Surveillance Network programme between 2011 and 2017.

4. Conclusions

Degree of achievement: Deliverable D.4.6. has been achieved.

Scientific and technical dissemination activities:

- Tzu-yen Teng, K.; Aerts, M., Jaspers S., Lopez G., Saez, J.L., Alvarez, J. Patterns of antimicrobial resistance in *Salmonella* isolates from fattening pigs in Spain (manuscript in preparation)
- Aerts M., Tzu-Yun Teng K., Jaspers S., Alvarez J. A new multicategory logit model for AMR data (manuscript in preparation).

Main conclusions:

The results obtained demonstrate the power of multivariate statistical methods such as Bayesian network analysis and hierarchical clustering in combination with spatial and temporal analyses applied to phenotypical AMR data for generating valuable insights about patterns of and associations between AMR phenotypes. Besides spatial and temporal trends in the percentage of isolates being resistant to various antimicrobials, we also found pairwise relationships between antimicrobials and patterns of resistotypes. The existence of serotype-specific AMR patterns for serotypes of public health concern in *Salmonella* isolates in pigs in Spain allow the generation of hypotheses on their possible genetic background.