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Detection of *Salmonella enterica* Serovar Rissen in Slaughter Pigs in Northern Ireland

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Abstract

Salmonella enterica serovar Rissen has been recognised as a common serovar in humans and pigs around the world. This study investigated *S.* Rissen prevalence in pigs slaughtered in Northern Ireland additionally looking at antibiotic susceptibility profiles, genetic profiles and plasmid profiles to provide information on an emerging non-typhoid *Salmonella* serotype with the potential to cause disease in humans. *S.* Rissen were isolated on five separate sampling occasions from both the boning hall and slaughter line of a randomly selected single pig abattoir in Northern Ireland (NI). Following antibiotic susceptibility testing against 16 antibiotics, all *S.* Rissen isolates were identified as susceptible to 15 antibiotics but resistant to tetracycline (R-type: Te). Of the 29 *S.* Rissen, 27 were isolated from pigs originating in NI and two *S.* Rissen were isolated from pigs originating in the Republic of Ireland (RoI). The combined results of the PFGE and plasmid profiling analyses were capable of subdividing the *S.* Rissen isolates into three distinct groups. The data suggests that *S.* Rissen is an emerging serovar in Northern Ireland and continued surveillance of this serovar is warranted as it has the potential to cause disease in humans.

Keywords

Antibiotic Susceptibility Testing, Pulsed Field Gel Electrophoresis, Plasmid Profiles

1. Introduction

The most recent reports identify *Salmonella* as the most common causative agent in food-borne outbreaks with known origin in the European Union (EU) [1]. The most recently published European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC) report identified *Salmonella enterica* serovar Rissen amongst the 20 most frequent serovars asso-

ciated with cases of human Salmonellosis and as one of the top 10 serovars associated with pig meat in the EU [2].

S. Rissen is a commonly reported serotype around the world and it is amongst the top three serotypes found in pigs and pork products in Europe and Southeast Asia [3]. Numerous studies have identified *S.* Rissen in slaughtered pigs in Portugal [4] and within pig fattening units and from slaughter-age pigs and Spain [5] [6] [7] [8] [9]. There have been further reports of *S.* Rissen in pig abattoir studies in Italy [10], Belgium [11], the Netherlands [12], Portugal [13] as well as in breeding herds in the UK [14] and in finishing pigs in Northern Ireland [15], thus demonstrating its' increase in European pig herds.

Worldwide *S.* Rissen is considered to be to be one of the most prevalent causes of human salmonellosis. In the USA in 2009 *S.* Rissen was responsible for a major *Salmonella* outbreak infecting more than 80 people over four states [16] and [17]. *S.* Rissen infections in humans have been reported in Ireland, Demark and the UK [18] [19] [20]. This clearly demonstrates that pigs continue to act as a carrier/host of *S.* Rissen infection and hence a potential reservoir of human infection. The risk of salmonellosis in humans coupled with the rise of multi-drug resistance in *Salmonella* highlights the need for the continued surveillance of emerging *Salmonella* serovars including *S.* Rissen. This study aims to add to previous research within the pig industry whilst adding to the continued assessment of the *Salmonella* serovars in pigs. This study investigated *S.* Rissen isolated from pigs slaughtered in Northern Ireland additionally looking at antibiotic susceptibility profiles, genetic profiles and plasmid profiles to provide information on an emerging non-typhoid *Salmonella* serotype with the potential to cause disease in humans.

2. Materials and Methods

2.1. Sampling

Sampling was carried out over an 18 month period. In all 405 samples were taken at the randomly selected single (out of seven) abattoir. For the oyster cut 25 - 50 g trimming was removed from the 50:50 product tray (50:50 is a half fat half pork trimming from the oyster cut) and transferred to a sterile jar (Trafalgar Scientific (CON7764) Leicester, UK). For faecal and caecal sampling the viscera was removed from the pig's abdominal cavity and following separate incisions into the rectum and caecum approximately 30 - 50 g of sample was removed and placed into sterile jars. Carcass swabs were taken from four EU approved swabbing points identified on the pig's carcass, jowl, belly, back and ham (EU directive 471/2001/EU) using EnviroSponge™ USDA approved cellulose sponges [Biotrace International (BP-133ES) Bridgend, UK]and placed in sterile bags provided with the sponge and labelled.

All samples were examined for the presence of *Salmonella* according to ISO 6579. Briefly 25 g of sample was transferred into 225 ml ISO Buffered peptone Water [BPW, Oxoid (CM1049) Basingstoke, UK], The sample and ISO BPW was transferred to a stomacher bag [Seward (BA6141) Worthing, West Sussex,

UK] and stomached for 1 min (Seward Tekmar Stomacher 400, Seward, Worthing, West Sussex, UK). The sample was incubated at 37˚ for 18 - 20 hours. Controls comprised *S.* Nottingham NCTC 7832 (positive control) and *Escherichia coli* NCTC10418 (negative control). There followed selective enrichment in Rappaport-Vassiliadis Soya (RVS) broth [Oxoid (CM0866) Basingstoke, UK], plating onto selective Xylose Lysine Decarboxylase [Oxoid (CM0469) Basingstoke, UK] and Brillant Green Agar [Oxoid (CM0263) Basingstoke, UK] followed by performance of confirmatory tests on suspect colonies.

All suspect *Salmonella* were sent to Agri-Food & Biosciences Institute (AFBNI), Newforge Lane, Belfast, Northern Ireland for serotyping. All resulting *Salmonella* were subsequently sent to The Laboratory of Enteric Pathogens, HPA, Collindale, UK to be phage typed using a standard set of the typing phage and following the Collindale scheme.

2.2. Antibiotic Susceptibility Testing

Resistance profiling used BSAC's disc diffusion method (http://www.bsac.org.uk/wp-content/uploads/2012/02/Version-8-January-2009.pdf) according to established protocols [21]. All *S.* Rissen, were tested for their *in vitro* sensitivity to 16 different antibiotics. The antibiotics and their concentrations were as follows; Amikacin 30 µg (AK), Ampicillin 10 µg (AMP), Amoxycillin & Clavulanic Acid 30 µg (AMC), Apramycin 15 µg (APR), Cefotaxime 30 µg (CTX), Ceftazidime 30 µg (CAZ), Chloramphenicol 10 µg (C), Ciprofloxacin 1 µg (CIP), Compound Sulphonamides 300 µg (Su), Furazolidone 15 µg (FR), Gentamicin 10 µg (Cn), Nalidixic Acid 30 µg (NA), Neomycin 10 µg (N), Streptomycin 25 µg (S), Sulphamethoxazole/trimethoprim 25 µg (SXT) and Tetracycline 10 µg (T). Minimum inhibitory concentration (MIC) tests were carried using M.I.C. Evaluators (Oxoid, Basingstoke, UK) where resistance was identified. Multidrug resistance was defined as having a resistance pattern to five or more antibiotics.

2.3. Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to the CDC PulseNet protocol [22] with some minor modifications. *Salmonella* isolates were streaked onto Tryptone Soya Agar [TSA, Oxoid (CM0131) Basingstoke, UK] and incubated at 37˚C for 18 - 20 hours. *Salmonella* isolates from TSA were resuspended in 2 ml of cell suspension buffer (CSB) (100 mM Tris: 100mM EDTA, pH 8.0) creating at attenuance of approximately 1.35 at λ 610 using a spectrophotometer. 10 µl of Proteinase K (20 mg/ml stock) [Sigma-Aldrich (P6556) Pole, Dorset, UK] was added to 200 µl of *Salmonella* suspension and gently mixed. 200 µl of molten 2% SeaKem Gold agarose [Cambrex (BMA 50150) Wokingham, Berkshire, UK] was then added to this suspension. *S.* Branderup H9812 was used as the reference strain. The bacterial/agarose mixture was then dispensed into PFGE disposable moulds [BioRad (170-3713) Hemel Hempstead Hertfordshire, UK] and allowed to solidify for approximately 10 minutes. The freshly moulded plugs were then transferred to

tubes containing 1.5 ml of CSB and stored at 4˚C. For cell lysis the plugs were placed in a 1.5 ml eppendorf containing 1 ml Cell Lysis Buffer-CLB (50 mM EDTA, pH 8, 1% sarcosyl) and 40 µl of Proteinase K solution (20 mg/ml) and incubated in a shaking incubator at 54˚C for 2 hours. The plugs were then washed in series of steps. Firstly, in a Polypropylene Super Clear™ 50 ml sterile tube, [Sarstedt (SLS8106) Leicester, UK] containing 10 - 15 ml of deionised waterin a shaking incubator at 50˚C for 10 minutes and following subsequent washing steps were placed in fresh TE buffer and stored at 4˚C until restriction digestion. The restriction enzyme *Xba*1 [Sigma-Aldrich (R7260) Poole, Dorset, UK] was first diluted 1:10 with dilute SuRE Cut buffer H [11417991001; Roche, UK] and 100 µl of this restriction mixture were added the micro-centrifuge tube containing the agarose plugs. Both sample and control plug slices were gently mixed and incubated for two hours at 37˚C.

A 1% agarose gel was prepared using SeaKem® Gold agarose [Cambrex (BMA50150) Woking, Berkshire, UK]. The gel plugs were then overlaid with a small quantity of molten SeaKem® Gold agarose [Cambrex (BMA50150) Woking, Berkshire, UK] and allowed to solidify. PFGE was conducted in a CHEF DR II chamber (BioRad, Hertfordshire, UK) containing fresh 0.5 M Tris Borate EDTA (TBE) running buffer [Gibco, (15581-044) Paisley, UK] under the following conditions: Initial switch time 2.2 s; Final switch time 63.8 s; Start Ratio 1.0; Voltage 200 V; Run time 19 - 20 hours at 14˚C. After staining for 20-30 min (in 40 µl ethidium bromide (EtBr) [Sigma-Aldrich (160539) Poole, Dorset, UK] and de-staining in sterile water for 60 min the gel image was then visualised and captured using a transillumination table (AlphaImagerTM 2200, Alpha Innotec, CA, USA). All PFGE gel images were converted to TIFF files prior to data analysis. Dendrograms and cluster analysis were created using Bionumerics 5.1 software (Applied Maths, Belgium). Similarity analysis was performed using Dice coefficient, and clustering was created using the unweighted pair group method with arithmetic means (UPGMA). When isolates were found to be indistinguishable upon XBa1 digestion and additional enzyme Spe1, was used to improve discrimination.

2.4. Plasmid Profiling

Plasmid profiling was carried out using a modified version of the Kado and Liu method [23] [24]. A single colony was inoculated into 3 ml of Brain Heart Infusion [Oxoid (CM1032) Basingstoke, UK] and following incubation at 37˚C for 18 - 20 hours 1ml of the culture centrifuged at 6600 G for two minutes. Cells were lysed by addition of 100 µl of lysis buffer (3% SDS in 50 mM Tris, pH 12.6). The sample was incubated at 56˚C for 30 minutes in a pre-heated heat block, followed by a chloroform extraction. The upper aqueous layer was transferred to a 1ml eppendorf. Loading dye (6 - 8 µl) was added to each sample and vortexed (sample stored at −20˚ for later analysis). Each sample (12 µl) was loaded onto a 0.7% agarose gel with a super-coiled ladder [Sigma-Aldrich (D5292) Poole, Dorset, UK] as molecular marker. Electrophoresis run-25 volts for four hours

using a RunOne electrophoresis system (EmbiTec, CA, USA). The gel matrix was then stained using EtBr [Sigma-Aldrich (160539) Poole, Dorset, UK]. The resulting bands were viewed using a transilluminator (AlphaImagerTM 2200, Alpha Innotech Corp., USA).

3. Results

In all 29 *S.* Rissen, were isolated from the abattoir, 21 of which were isolated from pork cuts in the boning hall, while four came from the rectal contents of pigs (post-slaughter), two were isolated from caecum contents of pigs (post-slaughter) and one was from a carcass swab (post-chill). The *Salmonella* were isolated over an 18-month period on five separate sampling occasions. Two of the pigs positive for *S.* Rissen in terms of rectal samples were of Republic of Ireland (RoI) origin, the remaining *S.* Rissen were isolated from pigs originating in Northern Ireland. *S.* Rissen accounted for 59% of the *Salmonella* identified, followed by *S. typhimurium* (21%), *S. panama* (17%) and *S. meleagridis* (3%).

3.1. Antibiotic Susceptibility

All 29 *S.* Rissen isolates exhibited resistance to only a single antibiotic-tetracycline at a concentration of 10 μg/l. The MIC for Tetracycline was determined to be >256 μg/ml. No resistance to the fluoroquinolone antibiotic ciprofloxacin or to the third generation cephalosporin antibiotics cefotaxime and ceftazidime was observed.

3.2. PFGE Analysis Using Xba1 and Spe1

Consistent and unambiguous Xba1-PFGE profiles were generated from 23 of the 29 isolates and analysis of these identified 19 that displayed a common profile consisting of 15 bands which ranged in size from 20 kb to 668 kb approximately. These 19 *S.* Rissen were designated cluster A and all presented a similarity higher than 96% - 97% using the DICE co-efficient. The remaining four *S.* Rissen displayed a common profile consisting of 16 bands which ranged in size from approximately 20 kb to 668 kb. These were designated cluster B and represented a similarity of approximately 97%. As both cluster A and B generated such similar Xba1 profiles further PGFE analysis using the restriction enzyme Spe1, as an alternative to Xba1, was conducted on representative *S.* Rissen from both cluster A and cluster B (**Figure 1**). Spe1 generated RFLP profiles that were identical for all *S.* Rissen typed. This analysis when combined with that of the Xba1 profiles and analysed using the BioNumerics 5.1 software (Applied Maths, Belgium) indicated that all the *S.* Rissen isolates from the pig abattoir were a clonal match. No further genetic differences were observed.

3.3. Plasmid Profiling

All 29 *S.* Rissen isolates were observed to have a low molecular weight band plasmid of approximately 4.650 kbp. A second low molecular weight plasmid of approximately 2.750 kb in size was found to be present in three out of the 29

isolates (Figure 2). All three of these *S.* Rissen that harboured the 2.750 kbp plasmid were recovered on a single sampling visit and were isolated from pigs originating in NI. Three other *S.* Rissen isolates were recovered that did not harbour the 2.750 kbp low molecular weight plasmid (Figure 2). The three *S.*

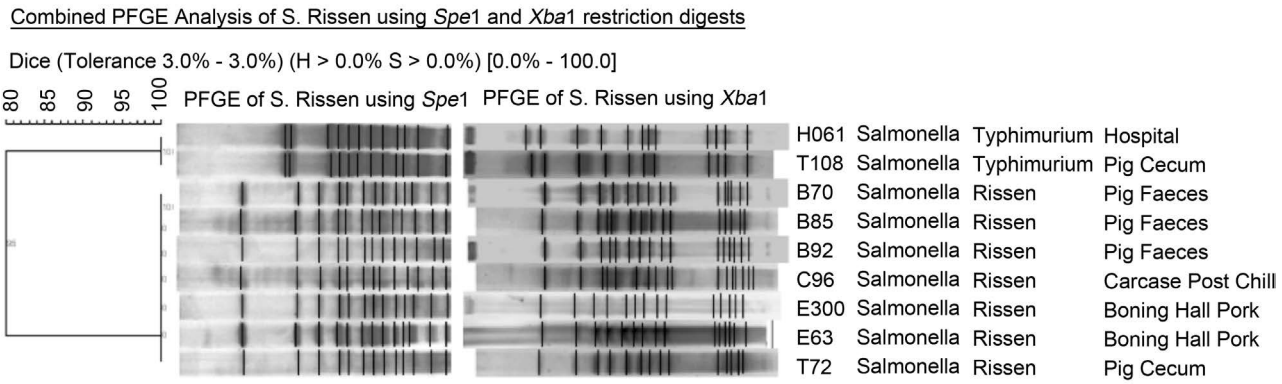

Figure 1. Pulse Field Gel Electrophoresis analysis of 7 of the *S.* Rissen isolated from a NI abattoir restricted with both SpeI and XbaI and the dendogram comparing the cluster analysis based on the combined macro-restriction profiles is shown. The 7 *S.* Rissen isolates: B70 (Pig faeces), B85 (Pig Faeces), B92 (Pig Faeces), C96 (Carcass Post Chill), E300 (Boning Hall pork), E63 (Boning Hall Pork), T72 (Pig Caecum). Two *S. typhimurium* isolates: H061 (laboratory isolate) and T108 (Pig Caecum) have been included for comparison purposes. The numbers on the horizontal axis represent the percent similarly between isolates based on dice coefficient and UGPMA clustering.

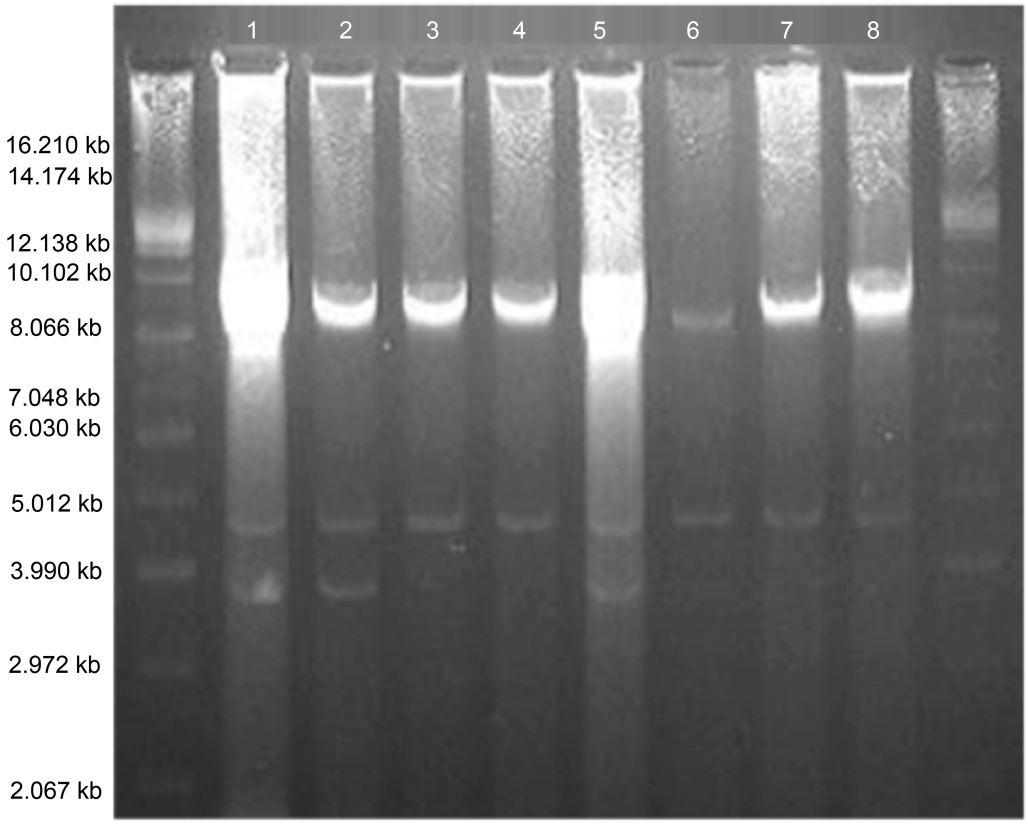

Figure 2. Plasmid profiles of *S.* Rissen isolates. Lanes 1 - 5 (*S.* Rissen isolates T70, T71, T72 from Pig Caecum). Lanes 6 - 7 (*S.* Rissen isolates B70, B71 from Pig Faeces) and lane 8 (*S.* Rissen C96 from a Carcass Swab). A low molecular weight plasmid (4650 KB) is observed in lanes 1, 2 and 5.

Rissen isolates displaying two plasmids were not the same isolates as the three *S.* Rissen observed to contain the extra band Xba1-PFGE in cluster B. The identification of two plasmid profile types significantly enhanced the differentiation of *S.* Rissen that were genetically dissimilar. When the genotyping data obtained through plasmid profiling is combined with that obtained by PFGE three different *S.* Rissen genetic profiles can be observed.

4. Discussion

All 29 *S.* Rissen exhibited resistance to a single antibiotic tetracycline (T) while displaying no resistance to the other 15 antibiotics at the concentrations tested in this study. Of the *S.* Rissen isolated from pigs in Europe, tetracycline is the most common resistance phenotype [7] [19] [25]. Garcia-Feliz [26] investigating the antibiotic resistance of a panel of 114 *S.* Rissen isolates, all originating from pigs, recorded that over 50% of isolates were resistant to tetracycline alone. This study supports other research that multidrug resistance is less common amongst *S.* Rissen isolates when compared to the antibiotic resistance characteristics of *S.* Typhimurium isolates [19] [26]. However, *S.* Rissen displaying multidrug resistance has been recorded including a study conducted in Spain which identified 48 of 114 S. Rissen isolates as MDR ranging from four to nine antimicrobials [26] and Thailand where *S.* Rissen displayed resistance from one to five antibiotics [27].

PFGE utilizing the restriction enzyme Xba1 resulted in two distinctive PFGE profiles among the 23 *S.* Rissen which were designated cluster A and cluster B. The remaining profiles were identical to the profiles observed for Cluster A and the *S.* Rissen isolates in cluster B that contained the extra band were still categorised as closely related [28]. The three *S.* Rissen in cluster B were isolated from pigs that were exported from the Republic of Ireland (RoI) for slaughter in NI. Two of the three *S.* Rissen isolated from these RoI reared pigs were recovered from faecal samples suggesting that these pigs may have been harbouring this *S.* Rissen clone on entering the abattoir.

PFGE and Plasmid profiling were used to establish the genetic diversity amongst the *S.* Rissen and suggests that the 23 *S.* Rissen, although not 100% identical, were all closely related. Xba1 generated PFGE images for *S.* Rissen from pigs and pork published by [4] [10] appear to have a common primary set of Xba1 generated restriction fragments consisting of approximately 15 restriction fragments similar to this study suggests that a common Xba1 pulsotype may exist for *S.* Rissen throughout Europe but further investigations would be necessary to confirm this.

5. Conclusion

PFGE identified two distinct profiles while plasmid profiling determined that three of the *S.* Rissen categorised as 100% clonal by PFGE actually carried an extra low molecular weight plasmid and of the 16 antibiotics tested against all 29 *S.* Rissen isolates, resistance to tetracycline was the only resistance phenotype ob-

served. The prevalence of this serovar is of some concern given that *S*. Rissen is capable of plasmid insertions and there may be a propensity for increases in *Salmonella* antibiotic resistance phenotypes. The literature would suggest that pigs are one of the main reservoirs of *S*. Rissen and it is likely that increased multidrug resistance in this serovar will see an increasing number of human cases. The continued surveillance of *S*. Rissen in pig herds is warranted.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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