Prevalence of antibiotic resistance genes in faecal samples from cattle, pigs and poultry

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ABSTRACT: Antibiotic resistant bacteria can be easily isolated from the faeces of cattle, pigs or poultry. However, whether the production of different farm animals is associated with a higher or lower prevalence of antibiotic resistance is not clear. In this study we therefore used real time PCR for the quantification of antibiotic gene prevalence in the DNA purified from the faeces of farm animals. First we showed that experimental streptomycin therapy of 12-week-old chickens and 46-week-old hens significantly increased the relative prevalence of *strA* and *sul2* genes though this did not necessarily indicate an absolute increase of *strA*-encoding bacteria. Next we quantified antibiotic gene prevalence in the DNA purified from the faeces of cattle, pigs and laying hens. The lowest prevalence of *strA*, *aadA*, *sul1*, *sul2*, *tet*(A), *tet*(B), *tet*(G) and *cat* genes was recorded in the intestinal contents of laying hens. In cattle and pig faecal samples, an intermediate prevalence of antibiotic resistance genes was observed with *strA* and *sul2* dominating by two logs over the remaining six tested genes. The differences in *strA* and *sul2* prevalence between cattle and pig microbiota were not significant whilst the prevalence of *strA* and *sul2* in laying hen microbiota was significantly lower than in the other two species. Cattle and pig production systems may therefore represent a more important reservoir of antibiotic resistant bacteria than laying hens.

Keywords: real time PCR; antibiotic resistance; farm animals; chicken; pig; cattle

Acquired antibiotic resistance in bacteria is reaching alarming levels. Nowadays, acquired resistance can be found in pathogenic bacteria as well as in commensal bacteria, despite the fact that the latter population is never intentionally targeted by antimicrobial treatment. The antibiotic resistance genes in commensal bacteria represent a reservoir from which these genes can be disseminated into different recipients even in the absence of antibiotic therapy (Nikolich et al. 1994). Even though antibiotic resistant clones are underrepresented in the microbiomes of healthy individuals, such clones are immediately positively selected for when an individual is subjected to antibiotic therapy.

Antibiotic resistance genes can be found associated with genetic elements of varying mobility. Conjugative plasmids can spread easily across bacterial populations (Sunde and Norstrom 2006; Hradecka et al. 2008), whilst genetic elements such as Salmonella genomic island 1 are mobilised for transfer at a significantly lower frequency (Doublet et al. 2005). Despite this, the mobility of elements transferring antibiotic resistance is not limited by species or genus and genetic elements with the same antibiotic resistance genes can be found across a broad range of different bacterial species.

A simple way to assess the range of antibiotic resistance in complex bacterial populations is by

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bacterial culture on nutrient agars with and without antibiotics followed by a comparison of the numbers of total and resistant colonies. For many purposes, this strategy is the most appropriate. However, it only provides information for the antibiotic resistance of bacterial species which are capable of growth under given culture conditions and not for those which may require different culture conditions. This is why culture-independent techniques such as quantitative real-time PCR are used for the characterisation of the prevalence of a particular gene in a given bacterial community (Yu et al. 2005; Chen et al. 2007). This experimental approach is especially useful for the quantification of antibiotic resistance genes in faecal microbiota, where the population is quite dense allowing for frequent horizontal gene transfer (Nikolich et al. 1994).

Antibiotic resistance poses a serious problem that is difficult to overcome. Prudent use of antibiotics and/or ecological farming may lead to a decrease in the prevalence of antibiotic resistance genes in bacterial populations (Young et al. 2009). However, although the recommendation of the prudent use of antibiotics may appear simple, the actual logistical and associated costs might be quite high (Salyers and Amabile-Cuevas 1997; Andersson and Hughes 2010). Animal production is commonly associated with large-scale use of antibiotics and is therefore considered as one of the major sources of new combinations of antibiotic resistance. Although it is quite simple to isolate multidrug resistant bacteria from the faeces of farm animals, this neither provides information on their quantitative representation, nor allows for the comparison between different farm animals. In this study we therefore tested whether some animal production systems represent reservoirs of antibiotic resistant bacteria more so than others. Furthermore, to avoid any bias potentially introduced by bacterial culture, we selected eight different target genes known to be responsible for antibiotic resistance and quantified their prevalence by real time PCR. In doing this we quantified and compared the prevalence of antibiotic resistance genes in the faecal microbiomes of cattle, pigs and egg-laying hens. We found that the prevalence of *strA* and *sul2* in cattle and pig faecal microbiota were significantly higher than in laying hen microbiota and cattle and pig production systems therefore represent a more important reservoir and source of antibiotic resistant bacteria than egg production.

MATERIAL AND METHODS

Sample characterisation. Altogether 34 cattle (24 meat type bulls and 10 cows of dairy cattle) and 39 pig samples were collected from the rectum immediately after slaughter in 2011. Seventy-seven laying hen samples were collected as fresh faecal droppings from four egg laying hen farms over a period of three years between 2009 and 2011 in the Czech Republic.

DNA purification and real time PCR. DNA was extracted using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen) and stored at –20 °C until use. Two sets of primers were used in real time PCR; those targeted at selected bacterial taxa and those targeted at selected antibiotic resistance genes. Taxon-specific primers were designed from the variable regions of 16S rRNA genes with PRIMROSE software (http://www.cardiff.ac.uk/biosi/research/biosoft/), and the specificity of the primers was verified using the RDP ProbeMatch program. The primers for quantification of the antibiotic resistance genes were designed using Primer3 software (Rozen and Skaletsky 2000). Finally, two primer pairs specific for the conserved regions of 16S rRNA genes (domain *Bacteria*-universal primer pairs) served to determine the total bacterial DNA present in these samples (Table 1). Real-time PCR was carried out using the QuantiTect SYBR Green PCR Kit (Qiagen) in a LightCycler LC480 thermocycler (Roche). After PCR, the Ct values of the genes of interest were subtracted from an average Ct value of amplifications performed with the domain *Bacteria*-universal primers (ΔCt). The relative frequency of each taxon or antibiotic resistance gene in the total bacterial population was finally calculated as $2^{-\Delta Ct}$.

Streptomycin therapy of chickens. Chickens were obtained from a farm with no history of antibiotic use. Daily water consumption was determined during the first days of their adaptation to the new environment and the determined daily water consumption was used to provide chickens with streptomycin in the drinking water at such a concentration that the daily uptake was equivalent to 15 mg of streptomycin per kg of body weight. In the first experiment, five 12-week-old chickens were administered streptomycin in the drinking water for seven days. In the second experiment, five 46-week-old hens were subjected to streptomycin therapy for two days only. Faecal samples

Table 1. List of primers used in this study

Primer	Target	Sequence 5'-3'	Reference
StrA-F	*strA*	CCAGTTCTCTTCGGCGTTAG	this study
StrA-R	*strA*	ACTCTTCAATGCACGGGTCT	this study
AadA-F	*aadA2*	CAG CCC GTC TTA CTT GAA GC	this study
AadA-R	*aadA2*	GAT CTC GCC TTT CAC AAA GC	this study
TetB-F	*tetB*	TACAGGGATTATTGGTGAGC	this study
TetB-R	*tetB*	ACATGAAGGTCATCGATAGC	this study
TetA-F	*tetA*	CGA TCT TCC AAG CGT TTG TT	this study
TetA-R	*tetA*	CCA GAA GAA CGA AGC CAG TC	this study
TetG-F	*tetG*	GTG TTC CCG ATT CTG TTG CT	this study
TetG-R	*tetG*	GAT TGG TGA GGC TCG TTA GC	this study
Cat-F	*cat1*	TCC ATG AGC AAA CTG AAA CG	this study
Cat-R	*cat1*	GGG AAA TAG GCC AGG TTT TC	this study
Sul1-F	*sul1*	GGATCAGACGTCGTGGATGT	this study
Sul1-R	*sul1*	GTCTAAGAGCGGCGCAATAC	this study
Sul2-F	*sul2*	CGCAATGTGATCCATGATGT	this study
Sul2-R	*sul2*	GCGAAATCATCTGCCAAACT	this study
16S_Bifido-F	*Bifidobacteriales*	GGTGTGAAAGTCCATCG	Juricova et al. 2013
16S_Bifido-R	*Bifidobacteriales*	ACCGGGAATTCCAGTCT	Juricova et al. 2013
16S_Clost-F	*Clostridiales*	GCGTTATCCGGATTTAC	Juricova et al. 2013
16S_Clost-R	*Clostridiales*	ACACCTAGTATTCATCG	Juricova et al. 2013
16S_Entero-F	*Enterobacteriales*	STGAGACAGGTGCTGCA	Juricova et al. 2013
16S_Entero-R	*Enterobacteriales*	AAAGGATAAGGGTTGCG	Juricova et al. 2013
16S_Lacto-F	*Lactobacillales*	CTTGAGTGCAGAAGAGG	Juricova et al. 2013
16S_Lacto-R	*Lactobacillales*	CACTGGTGTTCTTCCAT	Juricova et al. 2013
16S_univ-1F	all bacteria	GTGSTGCAYGGYTGTCGTCA	Maeda et al. 2003
16S_univ-1R	all bacteria	ACGTCRTCCMCACCTTCCTC	Maeda et al. 2003
16S_univ-2F	all bacteria	GAGGAAGGIGIGGAIGACGT	Tseng et al. 2003
16S_univ-2R	all bacteria	AGICCCGIGAACGTATTCAC	Tseng et al. 2003

were collected individually from each chicken. The first sampling was performed just prior to streptomycin administration (day 0) followed by sampling on days 1, 2, 3, 4, 7, 8, 9, 10, 11 and 14. The handling of animals in the study was performed in accordance with current Czech legislation (Animal protection and welfare Act No. 246/1992 Coll. of the Government of the Czech Republic).

Statistics. ANOVA followed by Tukey's post hoc test was used for the comparison of antibiotic resistance gene prevalence in the microbiome of different animal species. ANOVA followed by Dunnet's test post hoc test was used for evaluating the statistical significance in the experiments where streptomycin was administered to chickens.

RESULTS

Quantification of antibiotic resistance gene prevalence by real time PCR

The lowest antibiotic gene prevalence was recorded in the intestinal contents of laying hens. Real time PCR quantification indicated that *strA* or *sul2* was present in 20 or 8 out of 100 000 bacteria in the laying hen faecal microbiomes, respectively. In cattle and pig faecal samples, an intermediate level of prevalence for these two antibiotic resistance genes was observed (Figure 1). In the faecal microbiomes of these animals, *strA* and *sul2* dominated over the remaining six tested antibiotic re-

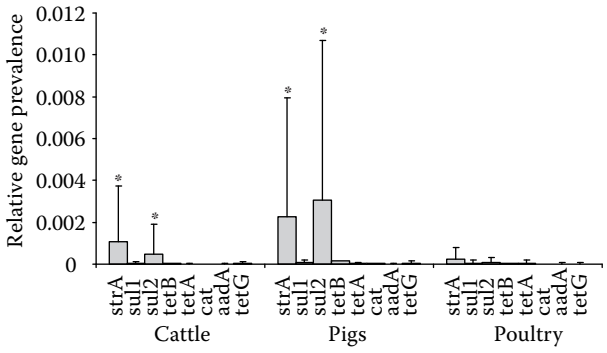

Figure 1. Real time PCR quantification of the prevalence of antibiotic resistance genes in faecal material from cattle, pigs and poultry. Asterisks indicate antibiotic resistance genes significantly differing in their prevalence of the same gene in poultry samples

sistance genes such as *sul1*, *tet*(A), *tet*(B), *tet*(G), *aadA* and *cat*. The genes *strA* and *sul2* were estimated to be present in 5 to 30 out of 10 000 bacteria indicating an approximately 10 times higher relative prevalence than in laying hen samples. When cattle, pig and laying hen samples were compared for the relative prevalence of *strA* and *sul2*, the differences in *strA* and *sul2* antibiotic gene prevalence between cattle and pig microbiota were not significant whilst the laying hen microbiota was a significantly less important reservoir of *strA* and *sul2* ($P < 0.05$) than microbiota of the former two farm animal species (Figure 1).

Quantification of key bacterial taxa present in analysed samples

Since we initially expected that the antibiotic resistance genes targeted in real time PCR were commonly found in *Enterobacteriales* (Sunde and Norstrom 2006; Karczmarczyk et al. 2011; Soufi et al. 2011), enrichment of some of the samples for *Enterobacteriales* could affect the final results. In the next experiment we therefore tested the composition of bacterial taxa (*Clostridiales*, *Lactobacillales*, *Enterobacteriales*, *Bifidobacteriales*) present in the faecal DNA by taxon-specific real time PCR. In cattle samples, *Clostridiales* dominated over the remaining orders. In pig samples, *Clostridiales* and *Lactobacillales* were present at a similar prevalence, significantly higher than *Enterobacteriales* or *Bifidobacteriales*. In laying hen samples, *Lactobacillales* dominated over the remaining three orders. The relative prevalence of *Enterobacteriales* reached 0.40% in the samples originating from the cattle, 1.04% in pigs and 0.80% laying hens (Figure 2). The *Enterobacteriales* prevalence among the samples therefore cannot explain the lower prevalence of *strA* and *sul2* in laying hen samples when compared with those from cattle and pigs.

Influence of streptomycin therapy on the presence of antibiotic resistance genes in the faeces of chickens

The different prevalence of antibiotic resistance genes observed in the cattle, pig and laying hen samples prompted us to test to what extent this could be influenced by a recently administered antibiotic therapy. To test this we treated chickens with streptomycin in two independent experiments. In the first experiment, chickens were treated for seven days whilst in the second experiment the antibiotic administration lasted for two days only. In both experiments, a rapid increase in the prevalence of *strA* and *sul2* genes was recorded. In addition, the prevalence of the *sul1* gene increased in the second experiment. The increase in *strA* and *sul2* prevalence reached statistical significance on day 2 and 4 when compared with day 0 in the first experiment. In the second experiment, the *sul2* and *sul1* prevalence increased significantly on day 3 and 4 when compared with day 0, respectively. Streptomycin therapy therefore not only increased the prevalence of *strA*-encoding streptomycin phosphotransferase responsible for the resistance to streptomycin, but

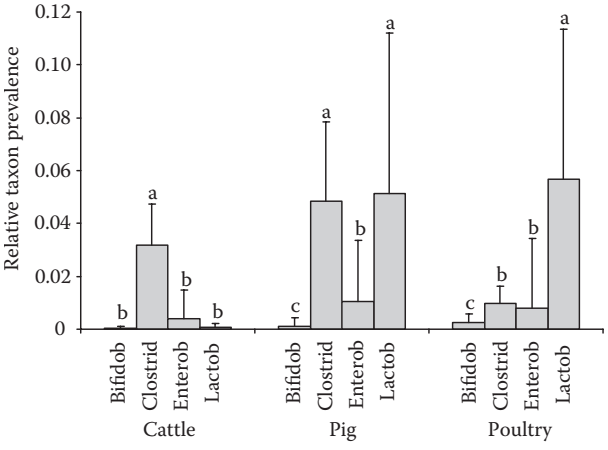

Figure 2. Taxon composition in faecal material from cattle, pigs and poultry determined by real time PCR. Indices indicate microbiota members not differing in prevalence from each other but differing from the remaining microbiota members within the same animal samples

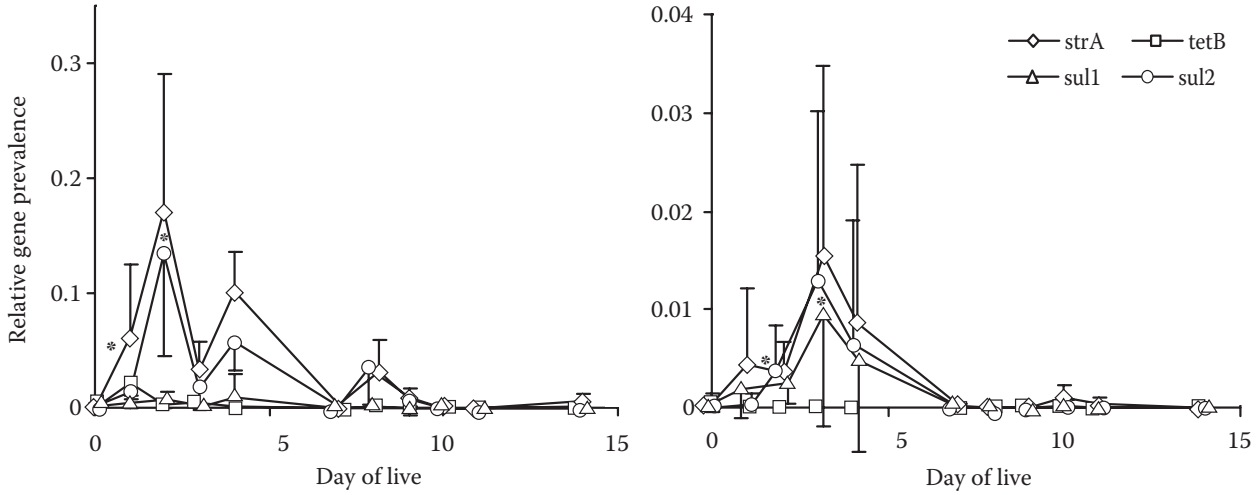

Figure 3. Influence of streptomycin therapy on the prevalence of antibiotic resistance genes in faecal DNA determined by real time PCR. Left panel, the first experiment with 7-day-long therapy. Right panel, the second experiment with 2-day-long therapy. Asterisks indicate a significant difference in gene prevalence when compared with day 0

resulted also in the co-selection of "non-target" genes such as *sul2* in the first experiment, and *sul1* and *sul2* in the second experiment, both encoding resistance to sulphonamides (Figure 3).

DISCUSSION

In this study we were interested in the prevalence of antibiotic resistance genes in faecal material from cattle, pigs and laying hens originating from the Czech Republic. When the farm animals were compared among each other, the highest prevalence of the antibiotic resistance genes targeted in this study was found in the faecal microbiota of pigs, followed by cattle and laying hens. There was a notable numeric difference between the pig and cattle samples, but this did not reach statistical significance. However, this difference corresponded with the observations of Yu et al. who reported a slightly higher prevalence of tetracycline resistance genes in pig faecal samples than in those from cattle (Yu et al. 2005). On the other hand, the prevalence of *strA* and *sul2* in the faecal microbiota of egg-laying hens was significantly lower than in pigs or cattle and therefore egg production can be considered as a lower risk for the selection and shedding of antibiotic-resistant bacteria.

The antibiotic resistance genes that were targeted consisted of those commonly present in *Enterobacteriales* (Faldynova et al. 2003; Hradecka et al. 2008; Havlickova et al. 2009). The expected association of the target genes with a particular order was the reason why we also monitored the presence of selected bacterial taxa in the collected samples. Since the prevalence of *Enterobacteriales* was similar in all the samples ranging from 0.4 to 1.08 %, this difference could not explain the difference in the antibiotic resistance gene prevalence.

In the second part of this study, we tested to what extent recent antibiotic therapy may influence the relative gene prevalence in faecal samples. Experimental streptomycin therapy in chickens increased the prevalence of the *strA* gene so that one or 15 out of 100 bacteria in the chicken faecal microbiomes harboured *strA* in their genome in the experiment 1 or 2, respectively. Interestingly, streptomycin therapy also increased the prevalence of genes coding for sulfonamide resistance in both experiments, which is likely due to the common presence of *strA* and *sul2* genes on the same genetic elements (Sunde and Norstrom 2006; Hradecka et al. 2008). However, it has to be noted that the increases reported in this study may not necessarily correlate with an absolute increase of *strA*-encoding bacteria. If streptomycin killed streptomycin-susceptible bacteria but left the *strA*-positive ones unaffected, the latter will increase in proportion but not in actual numbers. Interestingly, the remission of antibiotic resistance gene prevalence after therapy withdrawal was nearly as rapid as the increase immediately after the therapies, similar to the total microbiota restoration soon after antibiotic withdrawal as reported elsewhere (Antonopoulos et al. 2009; Jernberg et al. 2010; Videnska et al. 2013). This could be caused by a positive selection

of streptomycin-resistant bacteria not harbouring the *strA* gene dependent mode of resistance during the course of therapy and/or rapid multiplication of antibiotic-susceptible bacteria soon after streptomycin withdrawal. Unfortunately, this phenomenon makes the use of real time PCR quantification of these antibiotic resistance genes less suitable for assessment of recent antibiotic use in these animal species.

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