Final report club 5 joint research
Maximum 4 pages;

Title (no more than 100 characters) Molecular epidemiology of *bla*$_{CTX-M-1}$ plasmids in non-broilers *Escherichia coli* isolates from UK, Sweden and France
Participating partners: Anses (Lyon) / SVA / AHVLA
Project leader: Anses (Lyon)
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Proposal Summary (no more than 100 words): Genes encoding Extended-Spectrum Beta-Lactamases of the CTX-M group are highly prevalent, and plasmids play a key role in their spread. The *bla*$_{CTX-M-1}$ IncI1/ST3 plasmid is dominant in various animals in France. However, the situation in other countries is not known. The epidemiology of the *bla*$_{CTX-M-1}$ gene in Europe may differ between broilers and non-broiler animals such as cattle, horses and pets, as a consequence of major international exchanges (broilers) versus in-house antimicrobial selection (other animals). This project intents to highlight this issue in exploring the CTX-M-1 plasmid reservoir in UK, Sweden and France outside the broiler production.

Date started:	December 2013
Length of project	One year
End of project:	December 2014

Project Team		
Names	Institute	Role in project
Jean-Yves Madec	Anses Lyon	Both partners managed their own collection and final results were analyzed and compared
Stefan Börjesson	SVA	
Chris Teale	AHVLA	**Declined**

Research Questions
While the importation of birds may explane the suddent increase of ESBL/pAmpC rate in the broiler production in Sweden (see above), the *bla*$_{CTX-M-1}$ gene has also been recognized in *E. coli* isolates from other animal species in this country. Those animals include foremost horses, but also dogs and cattle, which were not associated with a strategy of animal importation. A similar situation is observed in UK where non poultry-associated CTX-M-1-producing isolates are available. In France, other CTX-M-1 producers from cattle, horses, pets were also collected since the first published study. Additional CTX-M-1 producers isolated between project submission and start will be included as well. The research question relies upon the nature and origin of the *bla*$_{CTX-M-1}$-carrying plasmids recovered from outside the broiler production. Presumably, these *bla*$_{CTX-M-1}$ plasmids reflect an autotochnous epidemiological picture of selection and dissemination of the *bla*$_{CTX-M-1}$ gene in animals. On the other hand, it cannot be ruled out that these *bla*$_{CTX-M-1}$ plasmids may (partly) derive from the broiler reservoir as well, similarly to France where identical *bla*$_{CTX-M-1}$ plasmids were found in poultry, cattle and horses. Characterizing the *bla*$_{CTX-M-1}$ plasmids outside the European broiler production is therefore the research question of the project. The comparison is also particularly relevant among countries with highly different rates of antibiotic usages, such as UK, Sweden and France.
Key Objectives:
To characterize the *bla*$_{CTX-M-1}$ plasmids from a collection of 100 CTX-M-1-producing *E. coli* isolates recovered from animals outside the broiler production (horses, cattle, pets) in UK (30 isolates), Sweden (30) and France (40).

To compare these plasmids in the three countries, and to existing data in France.
To determine whether common *bla*$_{CTX-M-1}$ plasmids are disseminated by dominant *E. coli* clones or not
Based on the data, to formulate hypotheses on the possible different routes of diffusion and selection of the *bla*$_{CTX-M-1}$ plasmids in animals in UK, Sweden and France depending on animal species/production

Brief workplan, including key milestones

The activities within the project are divided over 4 workpackages.
WP1 : Collection of isolates and harmonization of methods
Task 1.1: Definition of the final collection at beginning of the project.
Task 1.2: Harmonization of methods
WP2 : Molecular characterization of the CTX-M isolates
Task 2.1: Sequencing of CTX-M group 1 isolates where necessary (new isolates)
Task 2.2: Clonality of isolates (PFGE), Phylogrouping (PCR), MLST on relevant isolates (carriers of the same *bla*$_{CTX-M-1}$ plasmids)
Task 2.3: Replicon typing on native isolates
WP3 : Molecular characterization of the CTX-M-1 plasmids
Task 3.1: Conjugation /transformation experiments
Task 3.2: Replicon typing on transconjugants/transformants / S1-PFGE hybridizations with CTX-M and replicons probes
Task 3.3: Determination of plasmid subtypes (pMLST)
Task 3.4: Restriction Fragment Length Polymorphism (RFLP) on *bla*$_{CTX-M-1}$ plasmids and hybridization on RFLP gels
Task 3.5: Plasmid sequencing of one to three common *bla*$_{CTX-M-1}$ plasmids
WP4 : Comparison of the data
Task 4.1: Comparison of RFPL / plasmid subtypes / hybridization data
Task 4.2: Comparison of sequencing data of identical *bla*$_{CTX-M-1}$ plasmids
Task 4.3: Comparison with strain typing in case of identical *bla*$_{CTX-M-1}$ plasmids
Task 4.4: Publication of the results

Milestone No	Milestone title	Month
1	Strain collection and methods defined	1
2	Identification of CTX-M-1 isolates	3
3	Characterisation of plasmids and clones	8
4	Comparison of plasmids/clones	10
5	Publication of the results	12

Planned output.*(deliverables, to include possible publications and IP issues)*

Deliverable No	Deliverable title	Month
1	A European collection of CTX-M-1 *E. coli* producers outside the broiler production	3
2	Qualitative information on *bla*$_{CTX-M-1}$ plasmids and clones from animals in Europe outside the broiler production	9
3	Common peer-reviewed international publication submitted	12

Results (maximum 1,5 page)

As UK did finally not participate to the project, only CTX-M-1 producers from Sweden and France were included.

Sweden:
From 2009 to 2013, a total of 35 *Enterobacteriaceae* non-broilers isolates carrying $bla_{CTX\text{-}M\text{-}1}$ were available for further analyses at the SVA. These isolates were either received as clinical submissions at the National Veterinary Institute (horses (n=28) and dogs (n=3)), or isolated in the Swedish Veterinary Antimicrobial Resistance Monitoring programme (SVARM) (laying hens (n=3) or calves (n=1)). The isolates were *Escherichia coli* (n=33), *Hafnia alvei* (n=1), *Enterobacter cloacae* (n=1).
The $bla_{CTX\text{-}M\text{-}1}$ gene was found on various plasmid replicon types but the most common type identified was IncHI1, which was associated to isolates from horses. A majority of those plasmids belonged to plasmid MLST ST2. The remaining IncHI1 plasmids belonged to the highly related ST9.
In the current study, based on MLST, the *E. coli* isolates were found to be quite diverse. Some isolates belonged to the same ST or the same clonal cluster but in general, there was a broad variety with 21 different STs identified. These results indicate that an horizontal spread of IncHI1 plasmids have caused the dissemination of $bla_{CTX\text{-}M\text{-}1}$ among horses in Sweden between 2009 and 2013, rather than a clonal spread of a particular *E. coli* strain.
In addition, a majority of plasmids were shown to confer resistance to multiple antibiotics. To our knowledge, IncHI1 plasmids carrying ESBL-genes have not been described in the human settings in Sweden.
IncI1 plasmids were only found in *E. coli* isolates from one horse, one dog and two laying hens. The *E. coli* isolates from laying hens carrying IncI1 belonged to different sequence types, whereas the two plasmids found in them belonged to the same ST3 sequence type and also carried resistance to tetracycline and sulfamethoxazole. The *E. coli* isolate from the calf carried also the $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 plasmid but belonging to ST135, closely related to ST3. One horse also carried an IncI1 plasmid but the sequence type could not be determined due to the failure of pilL sequencing, but the other genes showed the same allele arrangement as seen in ST3. A couple of plasmids belonging to the IncF plasmid type were also identified, all in isolates of *E. coli* with different sequence types. In addition to one FII plasmid, two multireplicon plasmids (FIB+FIA and FIB+FII) were identified.

France:

Considering that Sweden had an over dominance of isolates from horses, a first similar collection was studied in France. Indeed, from 2011 to 2014, a total of 59 *Escherichia coli* isolates resistant to broad-spectrum cephalosporins were recovered from clinical horses and collected for further analyses through the Résapath network.

All *E. coli* isolates were from unrelated animals and proved to produce ESBLs by the synergy test. Among those, 46 produced ESBL belonging to the CTX-M group 1, including 35 CTX-M-1, 6 CTX-M-15, 1 CTX-M-32, 2 CTX-M-55, 2 non CTX-M. Next, the 35 CTX-M-1 producing *E. coli* isolates were selected for further plasmid characterization.

Among the various plasmid types carrying the $bla_{\text{CTX-M-1}}$ gene, IncH1 was found predominant. Other plasmid types were also found, which included IncI1, IncP and IncF.

As shown using Diversilab, the *E. coli* isolates were not clonal. Similarly to the situation in Sweden, this again argues for the horizontal spread of IncHI1 plasmids carrying $bla_{\text{CTX-M-1}}$ among horses in France, rather than the dissemination of a successful clone of *E. coli*.

Considering that a particular situation had been observed in France showing the wide spread of the $bla_{\text{CTX-M-1}}$ IncI1/ST3 plasmid, pMLST was performed on all IncI1 subtypes. All but one proved to belong to the ST3 subtype, the latter to ST135. Restriction Fragment Length Polymorphism (RFLP) carried out on those plasmids also demonstrated similar patterns.

A further set of 118 ESBL-producing cattle *E. coli* isolates recovered in 2012 was also included in the study, of which 59 produced CTX-M group 1 (including 36 CTX-M-1). The $bla_{\text{CTX-M-1}}$ gene was found on various plasmid replicon types but the most common type identified was IncI1 (n=18), all of them of the ST3 subtype. Other replicons types were IncF (n=4), IncN (n=7) and IncHI2 (n=2).

In conclusion, this study has produced numerous positives listed below:

1. a collection of 106 CTX-M-1 *E. coli* producers outside the broiler production was constituted (deliverable 1). This collection included mostly two different animal species, i.e. horses and cattle.
2. qualitative information on those $bla_{\text{CTX-M-1}}$ plasmids and clones was provided (deliverable 2). In particular, in both countries, clones were of poor contribution for the spread of the $bla_{\text{CTX-M-1}}$ gene and original data were obtained :
   - on the predominance of the IncHI1 plasmid carrying the $bla_{\text{CTX-M-1}}$ gene in horses, both in Sweden and France. This suggests that horses would be a reservoir of IncHI1 plasmids, which may further catch ESBL genes, such as the $bla_{\text{CTX-M-1}}$ gene. As $bla_{\text{CTX-M-1}}$ IncHI1 plasmids are rarely found in other animal species (including humans), this also indicates poor transfer efficiency between horses and other sectors.
   - on the $bla_{\text{CTX-M-1}}$ IncI1/ST3 plasmid recovered in Sweden and France from several animal species (horse, dog, laying hens, calves, …). Of note, this plasmid was also recently found dominant in CTX-M-1 *E. coli* producers in humans in France (Madec et al, AAC in revision), which questions its possible animal origin. Even though obtained on a small subset of animal species outside horses, these preliminary results of the $bla_{\text{CTX-M-1}}$ IncI1/ST3 plasmid also circulating in Sweden in animals would justify a large-scale European investigation to better precise its prevalence in the animal sector.
3. At this stage, a common peer-reviewed manuscript has not been submitted (deliverable 3), however there is no doubt that the results obtained are of international interest and publishable. Both groups produced several international publications on ESBLs during the last three years, which attest on their credibility to do so. The question of a common paper was discussed orally between Stefan Börjesson and Jean-Yves Madec during the last ECCMID 2015 meeting (Copenhagen). Considering that different angles for publication are possible, this has not been decided yet. The most immediate and original angle would be on IncHI1 plasmids spreading the $bla_{\text{CTX-M-1}}$ gene in horses both in Sweden and France.

In addition to the research results of the project, it was made possible : - to establish the method for plasmid MLST for IncI1 and IncHI1 at the Section of Antibiotics (Sweden) - to set up the Diversilab in the lab as an alternative typing method of PFGE (France)
Value of cooperation for club 5 institutes
A major output of the study includes the scientific exchanges that were strongly strengthened between SVA and Anses (Stefan Börjesson, Ulrika Grönlund, Marisa Haenni, Jean-Yves Madec), and we sincerely thank CoVetLab for this. As an example of fruitful exchanges, both groups decided to join in the same consortium in 2014 and 2015 (call in the Swedish equine sector, unfortunately rejected in 2014, re-submitted in 2015). Such exchanges will be anyway of major interest for future large-scale European calls.
Any other information
We regret the situation with UK, which was not predictable at the time of submission and acceptance of the project (internal re-organizations at APHA). Nevertheless, we think that the CoVetLab funding was scientifically fruitful for SVA and Anses. Further cooperation with APHA on the important topic of AMR was discussed at the 2015, 10th-11th CoVetLab meeting in Uppsala.
Evaluation

Deliverables met?	
Start of project	
Cooperation	
Advice towards new calls / new projects	