RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Impact of ceftiofur use on the dissemination of resistant enteric bacteria in finishing swine populations - **NPB # 10-138**

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Industry Summary:

The antibiotics Exceed and Excenel are two formulations of the cephalosporin drug ceftiofur that are commonly used to treat finishing pigs in the US. Our object was to understand the relationship between the use of ceftiofur and the spread of important cephalosporin resistance genes in swine finishing barns. To accomplish this we collected approximately 30 fecal samples from each of 50 finishing barns located in 5 US states. We tested the samples for the presence of *Escherchia coli* or *Salmonella* that were resistant to ceftiofur and other important antibiotics. Any isolates resistant to ceftiofur were then tested for specific genes that provide resistance to important cephalosporins known as $bla_{CTX\text{-}M}$ and bla_{CMY}. Of the 1495 total fecal samples that were tested, 109 (7.3%) from 25 barns were positive for *Salmonella*, but only 2 of those carried the bla_{CMY} gene and were resistant to ceftiofur. A total of 1174 (78.5%) of the fecal samples contained *E. coli* with the bla_{CMY} gene, but only 24 (1.6%) contained *E. coli* or other bacteria with the $bla_{CTX\text{-}M}$ gene. Pigs were commonly treated with ceftiofur as pigs in 41 (82%) of the barns had been exposed to ceftiofur prior to finishing in either farrowing or nursery barns. In addition, individual sick pigs in 24 (48%) finishing barns received ceftiofur for treatment. However, this reported ceftiofur use was not associated with the presence of *E. coli* carrying $bla_{CTX\text{-}M}$ or bla_{CMY}. This result suggests that the spread of resistant bacteria in swine finishing barns cannot be fully explained by simply measuring antibiotic use. Attempts to reduce resistance will likely require that complex relationships of factors be identified that promote the spread of resistant organisms and resistance genes.

Keywords:

Salmonella, *Escherichia coli*, antimicrobial resistance, beta lactamase, cephalosporins

Scientific Abstract:

Conveying resistance to penicillins and critically important cephalosporin drugs, the extended-spectrum β-lactamase bacterial resistance gene *bla*$_{CTX-M}$ was first described in US livestock in 2010 and characterized in dairy cattle populations in 2011. It has been hypothesized that the dissemination of *bla*$_{CTX-M}$ is a product of selection pressure from the veterinary use of third and fourth generation cephalosporins in food animals. Our objectives were to estimate the frequency and distribution of coliform bacteria harboring *bla*$_{CTX-M}$ in fecal flora of finishing swine populations in the US, and to characterize the CTX-M alleles, their plasmidic contexts, and the genetic diversity of the bacterial isolates. We also evaluated the association between barn-level ceftiofur use and the likelihood of recovering extended-spectrum cephalosporin resistant bacteria in these populations. We collected approximately 30 fecal samples from each of 50 finishing barns located in 5 US states. We recovered *E. coli* or *K. pneumoniae* containing *bla*$_{CTX-M}$ from 24 of 1495 (1.6%) fecal samples in 8 of 50 (16%) barns. Twelve of 30 (40%) samples from a single barn in MI produced *E. coli* containing *bla*$_{CTX-M-1}$ on IncN sequence type 1 plasmids. An additional 5 positive barns were in OH and yielded between 1 (3.3%) and 3 (10%) *E. coli* containing *bla*$_{CTX-M-1}$ or *bla*$_{CTX-M-15}$ on IncF plasmids. The 10 OH isolates also expressed chromosomally-mediated fluoroquinolone resistance. The remaining 2 isolates were *Klebsiella pneumoniae* carrying *bla*$_{CTX-M-1}$ recovered from 2 barns belonging to one company in Illinois. Pigs in most (82%) barns had received prophylactic ceftiofur treatment as piglets in either the farrowing or nursery facilities prior to the finishing phase of production. In addition, up to 8.7% of individual pigs in approximately half (48%) of finishing barns received therapeutic ceftiofur to treat acute disease. We were unable to detect an association between ceftiofur use and the probability of recovering *bla*$_{CTX-M}$ *E. coli*.

Introduction:

Extended-spectrum cephalosporin drugs are commonly used to treat invasive Gram negative infections, including salmonellosis in children. As a result, the World Health Organization considers these to be a "critically important" class of antimicrobial drugs for human medicine (World Health Organization (WHO), 2007). Ceftiofur is an extended-spectrum cephalosporin drug used only in veterinary medicine. However, its application to animals provides selection pressure on enteric flora that has been associated with the dissemination of resistant organisms in animal populations. It has been hypothesized that the veterinary use of ceftiofur is a public health risk because of the potential for the zoonotic food-borne transmission of resistant pathogens and resistance genes (Jörgensen, *et al*, 2007).

Bacterial resistance to extended-spectrum cephalosporins is conferred by enzyme production which inactivates the antimicrobial drug. In the US, the AmpC B-lactamase gene *bla*$_{CMY-2}$ disseminated widely in the enteric flora of US livestock populations following the introduction of ceftiofur (Mollenkopf, *et al*, 2012). In particular, MDR-*Salmonella* Newport harboring *bla*$_{CMY-2}$ emerged as an important pathogen of humans and livestock (Gupta, *et al*, 2003, Rankin, *et al*, 2002). More recently the *bla*$_{CTX-M}$ ESBL gene has emerged globally in both healthcare associated and community-acquired infections in humans as well as in companion and food animals (Lewis, *et al*, 2007, Wittum, *et al*, 2010). There is concern that enteric pathogens harboring *bla*$_{CTX-M}$ may emerge and disseminate in a similar manner.

bla$_{CTX-M}$ has been reported in populations of swine in Europe and in Asia. In Hong Kong, 2% of *E. coli* recovered from swine at slaughter harbored *bla*$_{CTX-M}$ (Duan, *et al*, 2006). Recovery of *E. coli* with *bla*$_{CTX-M}$ from two large swine operations in China increased from 0 to 10.5% coinciding with the introduction of therapeutic ceftiofur (Tian, *et al,* 2009). Others have reported the direct transmission of commensal *E. coli* carrying *bla*$_{CTX-M}$ between commercial swine and farm workers (Moodley and Guardabassi, 2009). A sampling of 20 retail meat

packages purchased from 7 supermarkets in Pittsburgh, PA identified a *bla*$_{CTX-M}$ isolate that was indistinguishable from that of a patient in a local healthcare facility (Doi, *et al*, 2010).

We previously reported the presence of *bla*$_{CTX-M}$ in fecal *E. coli* isolates from cattle in Ohio (Wittum, *et al*, 2010) and described their frequency and distribution in Ohio dairy herds (Mollenkopf, *et al*, 2012). In addition, we have reported the recovery of *Salmonella* harboring *bla*$_{CTX-M}$ from a swine diagnostic submission (Wittum, *et al*, 2012). However, the epidemiology of *bla*$_{CTX-M}$ in finishing swine populations has not been described. Therefore our objectives were to estimate the frequency and distribution of *Salmonella* and coliform species harboring *bla*$_{CTX-M}$ among finishing swine barns. We also characterized the genetic diversity of the isolates, their plasmids, and the *bla*$_{CTX-M}$ genes to understand the epidemiology of this resistance gene in swine populations. Finally, we investigated the association between antimicrobial use in the barn and the likelihood of recovering fecal bacteria harboring *bla*$_{CTX-M}$.

Objective:

To quantify the relationship between ceftiofur use in finishing swine and the dissemination of *Escherichia coli* and *Salmonella* spp. carrying plasmid-borne *bla*$_{CMY-2}$ and *bla*$_{CTX-M}$.

Materials & Methods:

Source of the Isolates. Our study population for cross-sectional sampling consisted of a convenience sample of 50 swine finishing barns located in 5 US states. In each barn 30 fresh fecal samples were collected from the floor and placed in individual sterile 50 ml conical tubes. When possible, based on barn design, non-adjacent pens were sampled with no more than 3 fresh fecal samples collected from each pen. When this sampling pattern could not be achieved, fresh samples were collected from throughout the barn to reduce the likelihood of multiple samples from the same animal. Samples from Ohio barns were transported to the laboratory at ambient temperature and processed on the day of collection. Samples from all other states were shipped by overnight courier at ambient temperature and processed on the day of arrival. Upon arrival to our research laboratory, each fecal sample was reduced to duplicate 4 g aliquots for the prospective culture of extended-spectrum cephalosporin resistant *E. coli* and *Salmonella* spp. In addition to the sample component of the study, a barn demographic data component detailing pig inventory, number of pens and antimicrobial use practices both before and while in the pigs were in this finishing barn was collected from barn owners or managers at the time of sample collection.

Bacterial culture. For the detection of *E. coli* resistant to extended-spectrum cephalosporins, one 4 g fecal aliquot from each sample was homogenized into 36 ml nutrient broth containing cefotaxime 2 *u*g/ml. After overnight incubation, this broth was streaked to MacConkey agar containing cefepime 4 *u*g/ml to identify isolates with the *bla*$_{CTX-M}$ phenotype, and to MacConkey agar containing cefoxitin 4 *u*g/ml to identify isolates with a *bla*$_{CMY}$ phenotype.
The remaining 4 g fecal aliquot was used for *Salmonella* spp. culture following our standard laboratory protocols. This aliquot was first enriched in supplemented Tetrathionate (TTB) and Rappaport-Vassiliadis R10 (RV) broths followed by differential selection on Xylose-Lysine-Tergitol 4 agar (XLT-4) (Heider, *et al*, 2009, Mollenkopf, *et al*, 2011). Bacteria from a single resulting black colony on XLT-4 was isolated on MacConkey agar and confirmed as *Salmonella* using standard biochemical reactions including triple sugar iron (TSI) agar, urea broth, and polyvalent antisera testing. Confirmed *Salmonella* spp. isolates were screened for extended-spectrum cephalosporin resistance by inoculation onto 2 MacConkey agar plates containing cefepime 4 *u*g/ml or cefoxitin 4 *u*g/ml with overnight incubation at 37°C.

Isolate Characterization. At the bacterium level, multiple methodologies were used to further characterized isolates with the expected phenotypes on selective media. Minimum inhibitory concentrations (MICs) to a panel of 26 antimicrobial drugs important to human and veterinary medicine were generated using a semi-automated broth microdilution system (Sensititre Sensitouch, TREK Diagnostic Systems, Cleveland, OH) following Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2008), providing a comprehensive antimicrobial resistance profile for each isolate. Pulsed-field gel electrophoresis (PFGE) genotyping (CHEF-DRIII, Bio-Rad Laboratories, Hercules, CA) was performed on total genomic DNA using *Spe* I (New England Biolabs, Ipswich, MA, USA) following CDC recommended procedures(Centers for Disease Control and Prevention, 1996 (updated 2000), Ribot, *et al*, 2006). The relatedness of strains was compared by examining banding patterns after electrophoresis, and applying generally-accepted criteria to assign levels of similarity (Tenover, *et al*, 1995). In addition, the isolates were clustered into genotypic groups using the Dice Coefficient Similarity Index via the Bionumerics software (Applied Maths, Kortrijik, Belgium).

Plasmid Characterization. The plasmid content of each isolate was visualized by electrophoresis using a standard procedure (Kado and Liu, 1981). Conjugation experiments (Gebreyes and Thakur, 2005) to establish the mobile nature of plasmids harboring $bla_{\text{CTX-M}}$ utilized filter mating of wild-type *E. coli* donors with a rifampicin and nalidixic acid-resistant derivative of the *E. coli* K12 MG1655 and a sodium azide-resistant *E. coli* KAM 32 derivative as recipient strains. Recipient acquisition of the expected plasmids and resistance genes was established with additional plasmid-profiling, $bla_{\text{CTX-M}}$ PCR, and sequencing of the resulting amplicons recovered from transconjugants. Individual plasmids were classified according to a standard replicon typing procedure that detects 18 replicon types based on incompatibility group loci(Carattoli, *et al*, 2005, Carattoli, *et al*, 2006) using PCR of boiled lysate template DNA. Plasmid multilocust sequence typing (pMLST) was applied to a subset of incompatibility group N plasmids using a set of 3 reported IncN primer sets (García-Fernández, *et al*, 2011). The resulting PCR amplicons were cleaned using USB ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, CA) following manufacturer's instructions and sequenced (GENEWIZ, South Plainfield, NJ) using analogous PCR amplification primers. Sequence type homology was detected using the Plasmid PubMLST database (http://pubmlst.org/plasmid/ accessed May, 2012). Direct comparisons of plasmid backbones were accomplished using restriction fragment analysis by digesting 10 µl of alkaline lysis extracted plasmid DNA overnight with 1 µl of *Acc* I (New England BioLabs) at 37°C.

Discrimination of $bla_{CTX\text{-}M}$ Alleles. PCR utilizing previously reported primer sets, was used to detect $bla_{\text{CTX-M}}$ as well as to screen for other important classes of β-lactamase resistance genes including CMY, TEM, SHV, and OXA (Lewis, *et al*, 2007, Ma, *et al*, 2005, Siu, *et al*, 2000). $bla_{\text{CTX-M}}$ PCR products were cleaned using USB ExoSAP-IT PCR Product Cleanup (Affymetrix) following manufacturer's recommendations. $bla_{\text{CTX-M}}$ Group 1 gene sequencing was accomplished using the corresponding PCR amplification primers by the Sanger method (GENEWIZ) and analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/).

Data Analysis. The relationship between owner-reported antimicrobial use and the probability of recovery of fecal bacteria carrying $bla_{\text{CTX-M}}$ was investigated using multivariable logistic regression procedures (Proc GENMOD, SAS version 9.2; SAS Institute Inc., 2008). Separate models were created to model the probability of a positive barn, or the probability of a positive sample using events/trial syntax. Generalized estimating equations were utilized to account for expected clustering within barns.

Results:

We obtained samples from swine finishing barns in Ohio (n=20), Kansas (n=10), Illinois (n-=10), Michigan (n=8), and Minnesota (n=2) during June through October of 2011. The expected 30 individual fecal samples were obtained from 47 of the barns, but only 29 were obtained from 2 barns and 27 from 1 barn for a total of 1495 fecal samples. The barns sampled housed a mean 1236 pigs (median =1135, stdev=510) in 35 pens (median=37, stdev=20.1) that had been in the barns for 98 d (median=100, stdev=33.1). Coliform bacteria harboring bla_{CTX-M} were recovered from 24 (1.6%) fecal samples representing 8 (16%) barns (Table 1). Of these, 22 were *E. coli* recovered from 5 barns in Ohio (n=10) and 1 barn in Michigan (n=12), and the remaining 2 isolates were *Klebsiella pneumoniae* recovered from 2 barns in Illinois. No coliform bacteria harboring bla_{CTX-M} were recovered from fecal samples collected in Kansas or Minnesota. Fecal *E. coli* harboring bla_{CMY-2} were recovered from 1174 (79%) samples representing all 50 barns, with the proportion ranging from 20% to 100% of samples within individual barns. *Salmonella* spp. were recovered from 109 (7.3%) fecal samples representing 25 (50%) barns. No *Salmonella* isolates carried bla_{CTX-M} but two *Salmonella* isolates recovered from separate barns belonging to the same company in Ohio each carried bla_{CMY-2}.

Pigs were commonly exposed to antimicrobial drugs as owners reported that only 3 (6%) barns did not receive antimicrobials while in the finishing barn. Pigs in 43 (86%) barns received antimicrobials in feed, most commonly tetracyclines (25 barns), lincosamides (13 barns), or macrolides (11 barns) with some (14 barns) receiving 2 or 3 different classes of antimicrobials in feed. Antimicrobials were also delivered in water to pigs in 7 (14%) barns, and individual sick pigs received antimicrobial therapy in 37 (74%) barns during the finishing period. Exposure to ceftiofur was also common as pigs in 41 (82%) barns were exposed to ceftiofur prior to finishing in either farrowing or nursery barns. In addition, individual sick pigs in 24 (48%) finishing barns received ceftiofur therapy with a median reported ceftiofur treatment rate of 1.2% of pigs and ranging from treatment of a single sick pig to a maximum of 8.7% of pigs treated in barns using ceftiofur. Reported antimicrobial use was not associated with the presence of *E. coli* carrying bla_{CTX-M} or bla_{CMY}.

E. coli with bla_{CTX-M} were recovered from 12 (40 %) of the 30 fecal samples from a single finishing barn in Michigan. PFGE of these isolates indicated that they represented a single *E. coli* strain. Plasmid analysis indicated that each of these isolates also carried an IncN plasmid of approximately 40 Kb bearing $bla_{CTX-M-1}$. Plasmid MLST identified that the IncN plasmids were each sequence type (ST) 1.

We recovered a total of 10 *E. coli* with bla_{CTX-M} from 5 finishing barns in Ohio, each barn belonging to the same company. Of these, 2 isolates from a single barn represented a single clonal strain of *E. coli*, each carrying $bla_{CTX-M-15}$ on IncFI plasmids. Seven isolates from 3 barns also represented the same clonal *E. coli* strain but harbored $bla_{CTX-M-1}$ on Inc FI plasmids. The remaining CTX-M *E .coli* isolate from this company displayed a unique pulsotype and an IncFIA plasmid bearing $bla_{CTX-M-1}$. Two *Salmonella* spp. carrying bla_{CMY-2} on IncA/C plasmids were recovered from two different barns in Ohio belonging to the same company.

We recovered a single *K. pneumoniae* isolate carrying $bla_{CTX-M-1}$ from two finishing barn belonging to the same company in Illinois. PFGE of these isolates indicated that they represented a single *K.pneumoniae* strain. The CTX-M-harboring plasmids that could not be typed using our incompatibility group typing procedure.

Discussion:

We recovered coliform bacteria harboring bla_{CTX-M} from 24 (1.6%) of the 1495 swine fecal samples, representing 8 (16%) of the 50 finishing barns sampled. Slightly higher recovery rates of *E. coli* carrying bla_{CTX-M} have been recently reported from fecal flora of dairy cattle in the US (Mollenkopf, *et al*, 2012). *E. coli* carrying bla_{CTX-M} were first described in fecal flora of US livestock in 2010 (Wittum, *et al*, 2010), although bla_{CTX-M} has

been previously recovered from livestock pathogens and commensal flora in both Europe and Asia (Moodley and Guardabassi, 2009, Horton, *et al*, 2011, Tamang, *et al*, 2011, Kameyama, *et al*, 2012). Recently, *Salmonella* spp. from US livestock diagnostic laboratory submissions, including one from a swine submission, were found to harbor $bla_{CTX\text{-}M}$ at a low frequency (Wittum, *et al*, 2012). Sporadic human cases of infection with CTX-M-producing *Salmonella* have been reported in the US (Sjölund, *et al*, 2008, Sjölund-Karlsson, *et al*, 2010, Sjölund-Karlsson, *et al*, 2011), but the zoonotic transmission of this resistance gene has not been described.

We observed no association between reported antimicrobial use, including therapeutic ceftiofur use, and the recovery of *E. coli* harboring $bla_{CTX\text{-}M}$. Similar results have been reported for dairy cattle where ceftiofur treatment rates were not associated with the probability of recovery of fecal *E. coli* with $bla_{CTX\text{-}M}$ (Mollenkopf, *et al*, 2012). Our data suggests that pigs are commonly exposed to ceftiofur either prior to or during the finishing phase of production. Ceftiofur treatment of individual pigs has been associated with a higher recovery of fecal coliform bacteria harboring $bla_{CTX\text{-}M}$ (Cavaco, *et al*, 2008), but similar associations between veterinary ceftiofur use and the recovery of $bla_{CTX\text{-}M}$ in livestock populations have not been reported.

PFGE analysis of our 22 $bla_{CTX\text{-}M}$-positive *E. coli* isolates indicated that clonal dissemination of a single strain occurred within barns, and among barns of the same company. We previously reported that strain homogeneity was common among *E. coli* isolates bearing $bla_{CTX\text{-}M}$ within dairy herds, although significant heterogeneity was observed in one dairy herd (Mollenkopf, *et al*, 2012). Pigs housed in finishing barns in the US are generally in a population-dense environment conducive to the sharing of enteric flora. Our result suggests that clonal expansion of resistant organisms and sharing of enteric flora among pigs is the primary mechanism of dissemination of $bla_{CTX\text{-}M}$ in US finishing barns.

We found that the $bla_{CTX\text{-}M}$ gene was carried by plasmids of incompatibility groups N and FI. The IncN plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ that we recovered from the single finishing barn in Michigan were all found to be ST1 by pMLST. The epidemic spread of ST1 IncN plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ in humans, livestock, and food has been reported in Europe (Naseer and Sundsfjord, 2011). In addition, ST1 IncN plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ have recently been recovered in the US from fecal *E. coli* of dairy cattle (Mollenkopf, *et al*, 2012) and from *Salmonella* diagnostic submissions from livestock (Wittum, *et al*, 2012). The mechanism for the pandemic dissemination of this unique combination of specific plasmid and resistance gene is not understood, but could result from prolonged and widespread antimicrobial selection pressure together with frequent and rapid movement of animals, people, and food products.

In addition to recovering $bla_{CTX\text{-}M}$, we also recovered *E. coli* with bla_{CMY} from 100% of the barns and from 79% of the individual fecal samples. Similar recovery rates have been reported in dairy cattle populations in the US, and those recovery rates appear to be increasing (Mollenkopf, *et al,* 2012, Heider, *et al*, 2009, Tragesser, *et al*, 2006). We also recovered two *Salmonella* from two finishing barns in Ohio that carried $bla_{CMY\text{-}2}$, suggesting that enteric coliform bacteria can serve as a reservoir of extended-spectrum cephalosporin resistance genes for pathogens. These resistant pathogens can contaminate fresh retail pork products (Mollenkopf, *et al*, 2011) and pose a risk to the public health.

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Table 1. Reported antimicrobial use among 50 swine finishing barns located in 5 US states.

	Barns (N=50)					
	Total		CTX-M (+)		CTX-M (-)	
Antimicrobial Use	N	prev	n	prev	n	prev
In feed	**43**	**0.86**	**7**	**0.88**	**37**	**0.88**
Bacitracin	2	0.04	0	0	2	0.05
Carbadox	7	0.14	3	0.38	4	0.10
Lincomycin	13	0.26	2	0.25	0	0.26
Macrolide	11	0.22	0	0	0	0.26
Tetracycline	25	0.5	5	0.63	20	0.48
Tiamulin	9	0.18	3	0.38	6	0.14
Virginiamycin	1	0.02	0	0	1	0.02
In water	**7**	**0.14**	**1**	**0.13**	**6**	**0.14**
Gentamicin	1	0.02	1	0.13	0	0
Lincomycin	1	0.02	0	0	1	0.02
Penicillin	3	0.06	0	0	3	0.07
Sulfamethazine	1	0.02	0	0	1	0.02
Tetracycline	3	0.06	0	0	3	0.07
Tiamulin	2	0.04	0	0	2	0.05
Parental	**37**	**0.74**	**8**	**1**	**29**	**0.69**
Florfenicol	1	0.02	0	0	1	0.02
Fluoroquinolone	3	0.06	0	0	3	0.07
Lincomycin	15	0.3	4	0.50	11	0.26
Penicillin	17	0.34	5	0.63	12	0.29
Tetracycline	7	0.14	1	0.13	6	0.14
Tylosin	5	0.1	0	0	5	0.12
Ceftiofur	**24**	**0.48**	**6**	**0.75**	**18**	**0.43**
Ceftiofur treatments	**741**	**0.012**	**47**	**0.01**	**694**	**0.01**
Pre-finishing exposure	**41**	**0.82**	**7**	**0.88**	**34**	**0.81**

Table 2. Recovery of extended-spectrum cephalosporin resistance genes bla and bla from 1495 fecal samples collected from 50 swine finishing barns located in 5 US states.

	Barns					Samples				
	Total	CTX-M (+)		CMY-2 (+)		Total	CTX-M (+)		CMY-2 (+)	
	n	n	prev	n	Prev	n	n	prev	n	prev
Total	50	8	0.16	50	1	1495	24	0.016	1174	0.785
State										
Illinois	10	2	0.2	10	1	300	2	0.007	291	0.97
Kansas	10	0	0	10	1	298	0	0	192	0.644
Michigan	8	1	0.125	8	1	240	12	0.05	178	0.742
Minnesota	2	0	0	2	1	60	0	0	59	0.983
Ohio	20	5	0.25	20	1	597	10	0.017	454	0.76