Salmonella enterica in imported and domestic day-old turkey poults in Egypt: repertoire of virulence genes and their antimicrobial resistance profiles

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Summary

Globalisation and international trade facilitate the rapid spread and transmission of foodborne pathogens. This study was designed to determine the serovars, distribution of virulence genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, *bcf*C) and antibiotic resistance profiles in salmonellae recovered from imported and domestic day-old turkey poults in Egypt. The prevalence of salmonellae in the imported poults was 4% (6/150): *S.* Enteritidis was the most frequent isolate (1.3%; 2/150), followed by Typhimurium, Virchow, Larochelle and a non-typeable strain, each with 0.7% (1/150) prevalence. The prevalence of salmonellae in the domestic poults was < 2% (2/150) and serotyping indicated a prevalence of 1.3% (1/150) for both Typhimurium and Altona. In polymerase chain reaction screening, the genes *invA*, *sop*B and *bcf*C were detected in all the Enteritidis, Typhimurium, Virchow, Larochelle, Altona and non-

typeable isolates (100%); the gene *gip*A was absent from all isolates. Carriage of *inv*A, *sop*B and *bcf*C among the Enteritidis, Typhimurium, Virchow, Larochelle, Altona and non-typeable isolates was associated with a core pattern of resistance to three antibiotics: streptomycin, nalidixic acid and chloramphenicol. The detection of *S.* Enteritidis, Typhimurium, Virchow, Larochelle, and Altona in turkey poults has important implications because these serovars are a significant cause of foodborne illness and enteric fever in humans.

Keywords

Antibiotic resistance – Poults – *Salmonella* Enteritidis – Typhimurium – Virchow – Virulence genes.

Introduction

Bacteria of the genus *Salmonella* are an important threat to both human and animal health worldwide (1). In the United States of America (USA), salmonellae are estimated to cause approximately 1,028 million illnesses annually, with 19,000 hospitalisations and approximately 400 deaths (2). Currently, more than half of all human salmonella infections in Denmark result from international travel and consumption of imported food (3). Worldwide, *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and *S.* Typhimurium cause the majority of human clinical cases (4); however, serovars of other nontyphoidal salmonellae are often more prevalent in particular countries and result in more severe infections and outcomes (3). Outbreak data have indicated that salmonellae are strongly associated with poultry and that turkey is one of the top three foods that contribute to *Salmonella* foodborne illness (17%) (5). Many *Salmonella* infections occur in people who handle contaminated turkey poults (6, 7, 8). Numerous *Salmonella* serovars have been isolated from turkey flocks; some may be predominant for many years in a certain region or country and then disappear to be replaced by others (9, 10, 11, 12, 13, 14). Such serovars have occurred in marked clusters, indicating persistent infection in hatcheries or introduction into one or more flocks at a certain time via infected day-old poults (12).

The widespread use of antibiotics as supplements for prophylaxis and growth promotion has influenced the selection of antibiotic-resistant strains of salmonellae at farm level during poultry production (15, 16). With particular reference to poultry production, most research on characterisation of antibiotic-resistant salmonellae has focused on the phenotypic and⁄or genotypic variations in resistant isolates, mainly from broiler chickens and turkeys (17, 18, 19). The dearth of information on turkey poults has resulted in the ecology of salmonellae in the turkey production system being poorly understood.

Investigation of the prevalence of *Salmonella* serovars that could pose threats to the turkey industry and human public health through importation of infected day-old turkey poults is important. To assess the virulence potential of *Salmonella* isolates from poults, the presence of 11 virulence-associated genes was determined: genes *inv*A, *avr*A, *ssa*Q, *mgt*C, *sop*B, *sii*D, *gip*A, *sod*C1 and *sop*E1 associated with *Salmonella* pathogenicity islands (SPIs), the fimbriae-related gene *bcf*C and the gene *spv*C from the *spv* operon associated with *Salmonella* pathogenicity. The repertoire of virulence genes in the isolates was determined, together with the antimicrobial susceptibility profiles.

Methods

Sampling

All samples from imported birds were sent to the Central Laboratory for Veterinary Quality Control on Poultry Production, situated in Giza, Egypt, which is part of the Agriculture Research Center of the Ministry of Agriculture. A total of 150 day-old turkey poults were randomly collected from 50 boxes (25 birds per box; 3 birds from each box) during the period 2012 to 2013. Faecal samples were collected from the 150 poults for culture and isolation of salmonellae. For comparison, faecal samples were obtained on day of hatch from 150 poults in two domestic breeder flocks (75 poults from each flock) at a single commercial hatchery (same line, all breeders in excellent health).

The sampling frame primarily covered holdings representing at least 80% of the total population of turkey poults, theoretically providing 95% confidence of detection of a 1% within-flock *Salmonella* prevalence, assuming an analytical method with 100% sensitivity.

Isolation procedure

Faecal samples were analysed for salmonella using culture methods as the gold standard in accordance with ISO-6579:2002 Annex D (20). Thus, 25 g of faeces were mixed with 225 ml of buffered peptone water (Oxoid Ltd, Hampshire, England) as pre-enrichment and incubated for 18 h at 37°C. Cultures were further enriched in selective Rappaport–Vassiliadis broth (Oxoid Ltd) and incubated for 24 h to 48 h at 42°C. The broth cultures were then streaked on Rambach agar (Merck, Darmstadt, Germany), xylose lysine desoxycholate agar (XLD, Oxoid Ltd) and Hektoen enteric agar (Oxoid Ltd) and incubated at 37°C for 24 h to 48 h. Presumptive *Salmonella* colonies were identified on the basis of Gram stain, catalase reaction, oxidase reaction and oxidation/fermentation of glucose. Gram-negative bacilli that were catalase positive, oxidase negative and capable of oxidation and fermentation of glucose were inoculated onto microtubes of API 20E strips (bioMérieux, Marcy L'Étoile, France) in accordance with the manufacturer's instructions. Bacteria were identified using the database API LAB Plus version 3.2.2. (bioMérieux). One *Salmonella* isolate from each positive sample was serotyped and subjected to further analysis using the tests listed below.

Salmonella serotyping

Typical *Salmonella* isolates were serotyped using antisera from Denka Seiken (Tokyo, Japan) and following the Kauffman–White serotyping scheme.

Antibiotic susceptibility testing using disc diffusion

Inhibitors of cell wall synthesis (aminopenicillins, colistin), protein synthesis (gentamicin, streptomycin, tetracyclines, chloramphenicol, neomycin) and nucleic acid synthesis (norfloxacin**,** ciprofloxacin,

trimethoprim-sulfamethoxazole, trimethoprim, nalidixic acid) were used in the inhibition tests. The panel of antibiotic discs used in panel screens represented eight drug classes. Antimicrobial susceptibility was tested according to the guidelines of the Clinical and Laboratory Standards Institute (21) for the disc diffusion technique using commercial discs (Becton, Dickinson and Company, Maryland, USA). Each isolate (*S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, *S.* Larochelle, *S.* Altona) was inoculated onto Muller–Hinton agar (Oxoid Ltd) and incubated at 37°C for 24 h. Zones of inhibition were measured to assess resistance or susceptibility. The following antimicrobials were chosen because of their common use in treating and preventing *Salmonella* infection in poultry and humans: ampicillin 10 mg, amoxicillin 20 mg, gentamycin 10 mg, neomycin 30 µg, streptomycin 10 mg, chloramphenicol 30 mg, colistin 10 mg, tetracycline 30 mg, trimethoprim 5 µg, sulfamethoxazole 23.75 mg + trimethoprim 1.75 mg. In addition, resistance to three broad-spectrum quinolones was assessed: ciprofloxacin 5 µg, norfloxacin 5 µg and nalidixic acid 30 mg. Antibiotic discs were placed on the surface of the inoculated agar plates (six discs/plate) and incubated for 24 h to 48 h at 37°C. After incubation the diameter of the halos was measured. Multidrug resistance (MDR) was defined as resistance to two or more antibiotics belonging to different antibiotic classes (22, 23).

Detection of virulence determinants

Virulence genes were detected using polymerase chain reaction (PCR) amplification as described previously (23, 24, 25). Amplicon sizes, gene functions, primers and conditions are shown in Table I. Isolates were screened for the presence of 11 virulence genes associated with pathogenicity in *Salmonella*: *inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C and *bcf*C.

Results

Prevalence and serotyping of salmonellae

The prevalence of salmonellosis in the imported poults was 4% (6/150) and serotyping of the isolated strains indicated that *S.* Enteritidis was the most prevalent (1.3%; 2/150), followed by Typhimurium, Virchow, Larochelle and a non-typeable strain at 0.7% (1/150) prevalence each. In contrast, the prevalence of salmonellae in the domestic poults was < 2% (2/150); the serovars of the isolated strains were Typhimurium and Altona, each with prevalence 1.3% (1/150).

Distribution of resistance to individual antimicrobial agents

In total, five serovars were detected among the seven typeable strains. Only two of the 14 antimicrobials (ciprofloxacin, colistin sulfate) were effective against all seven strains (Table II). The frequency of resistance to the rest of the antibiotics was variable, the resistance patterns showing great diversity. Overall, the five serovars were resistant to more than one antimicrobial: in descending order, *S.* Altona isolates showed resistance to six of the seven antimicrobial classes, *S.* Enteritidis isolates showed resistance to five classes (penicillins, fluoroquinolones, phenicols, sulfonamides, tetracyclines) and *S.* Typhimurium isolates showed resistance to four (aminoglycosides, fluoroquinolones, phenicols, tetracyclines). The Virchow and Larochelle isolates both showed resistance to aminoglycosides. The non-typeable strain isolated from imported poults was resistant to chloramphenicol, neomycin and tetracycline.

Virulence genes

All eight isolates (7 typeable, 1 non-typeable) were screened by PCR analysis for presence or absence of the 11 investigated virulence genes (Table III). The genes *invA* and *sopB* (carried on SPIs) and *bcfC* (fimbria-related) were present in all the isolates (100%). In contrast, *gip*A (encoding a Peyer's patch-specific virulence factor, GipA) was completely absent. The gene *spv*C, carried on a virulence plasmid,

was detected in Enteritidis and Typhimurium isolates from the imported turkey poults only. The gene *avrA*, located on SPI1, was present in the Enteritidis, Virchow and Larochelle isolates from imported poults and in the Altona isolate from domestic poults. The gene *mgt*C (carried on SPIs) was absent from the Typhimurium isolates from imported (1/6) and domestic (1/1) poults, whereas it was present in all the other serovars. In contrast, only the Enteritidis, Typhimurium and Larochelle isolated from imported poults tested positive for *sod*C1 (located on a bacteriophage).

Overall, six combinations of virulence genes were detected in the imported poults and two other combinations in the domestic poults (Table III). The isolates of *S.* Typhimurium showed two different virulence gene repertoires: the combination *inv*A, *sod*C1, *sop*B, *spv*C and *bcf*C in an imported strain and the combination *inv*A, *sop*B and *bcf*C in a domestic strain. Ten of the 11 virulence genes were associated with the isolates from imported poults, whereas in the Typhimurium and Altona isolates from domestic poults *sodC1*, *sopE1* and *spvC* were not detected.

Association of antimicrobial resistance phenotype with virulence-associated genes

The antimicrobial resistance patterns in the eight isolates in relation to the presence of the 11 virulence genes studied is shown in Table III. The two imported Enteritidis isolates, with identical virulence gene repertoires, showed different antibiotic resistance phenotypes: both were resistant to chloramphenicol, neomycin, nalidixic acid, tetracycline, trimethoprim and trimethoprim-sulfamethoxazole but only one was resistant to ampicillin and norfloxacin.

Discussion

As a zoonotic foodborne bacterium, *Salmonella* has reservoirs in various animals, including poultry, which are considered a reservoir for salmonellae and a potential cause of disease outbreaks in the human population. Turkeys are known to harbour this organism (26, 27) and among turkey production flocks in the European Union (EU)

in the period 2005 to 2007 up to 1.5% were positive for *S.* Enteritidis or *S.* Typhimurium and in 2007 approximately 8% of the EU turkey fattening flocks tested positive for salmonellae (28). The bacteria can be introduced into a turkey site potentially at all stages in the production pyramid and can be transmitted both vertically and horizontally (14). Turkey breeding flocks and hatcheries are critical sources of salmonellae, and it has been reported that flocks may remain infected with this organism throughout the growing period (29). Some of these *Salmonella* species are commonly implicated in human and animal disease but emerging strains are also gaining recognition. The annual list from the Centers for Disease Control and Prevention now includes exotic strain types not previously recognised (30).

Comparisons of the present study with others should be made with caution because of differences in monitoring schemes, study design, laboratory methods and reporting. It is clear from published reports and from the recent EU baseline survey for salmonellae in turkeys (31) that certain serovars are widely distributed and likely to reflect the international trade in breeding turkeys, day-old poults and contaminated feed ingredients. Newly hatched pullets are more susceptible to colonisation of the gastrointestinal tract by salmonellae during their first few days, through vertical transmission from infected parents or through horizontal transmission at the hatcheries during feeding, handling, poultry-house environment and transportation (1). It has been noted that *S. enterica* subsp. *enterica* serovars and their antibiotic resistance patterns are affected by the age of the birds (32).

The isolation of salmonellae in this study suggests that turkeys may act as a reservoir for these strains, which can be transferred to humans. Isolates from turkeys have been reported as having high levels of antimicrobial resistance (10, 33) and as being more frequently resistant than isolates from other livestock species (10, 34). The global increase in resistance to tetracycline and streptomycin has been observed in salmonellae of animal origin (18); this is not surprising as these two antibiotics are among those most commonly used in production of food animals worldwide (35). Both antibiotics have

been approved for use in turkey production in the USA (36), but their widespread use at farm level has been linked to the development of resistance (15). Several studies have reported that the resistance pattern for streptomycin is associated with that for tetracycline (15, 19). A similar observation was made in the present study, where one of the *S.* Typhimurium isolates showing resistance to streptomycin was also resistant to tetracycline, and co-resistance to these two antimicrobials was observed in *S.* Altona.

Salmonella strains of turkey origin are also often resistant to a variety of antimicrobials approved for use in poultry; these include tetracycline, chloramphenicol and aminoglycosides (9). Although resistance to ciprofloxacin, as observed by Logue *et al.* (13), was not found in any of the turkey isolates in the present study, resistance to chloramphenicol and nalidixic acid was detected. Some serovars, such as *S.* Typhimurium and *S.* Virchow, do not show resistance to nalidixic acid even though they may have been subjected to the same selective pressure (14, 15). Nevertheless, the relatively common resistance of turkey isolates to nalidixic acid (14, 19) is considered alarming, as fluoroquinolones are used for the treatment of invasive salmonellosis (15, 37). The increasing occurrence of quinolone-resistance in isolates from food animal sources has been reported as a matter of concern (38).

The occurrence and proliferation of antibiotic-resistant salmonellae in turkey poults may be the consequence of the practice of dipping hatching eggs in solutions containing antimicrobial agents (38, 39, 40) and/or the routine inoculation of day-old poults with antibiotics (39, 40, 41). In the present study, all the serovars showed total susceptibility to ciprofloxacin and colistin sulfate, which are widely used in other animal production environments for growth promotion and the treatment and prevention of disease and were included in the World Organisation for Animal Health List of Antimicrobials of Veterinary Importance at its 75th General Session in May 2007 (Resolution No. XXVIII) (42); they are also listed and categorised as critically important antimicrobials used in human medicine (43).

In the present investigation, 10 well-recognised virulence genes with implications for human health (*avr*A, *ssa*Q, *mgt*C, siiD, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, *bcf*C) were screened by PCR assay, based on previous studies in Europe, the USA, Mexico, Africa and Asia (3, 25). These virulence determinants have been shown to be widely distributed among isolates of salmonellae recovered from animals, birds and humans, but with some diversity (3, 25). The diversity in distribution could be explained by serovar specificity of virulence plasmids, such that not all plasmid-bearing serovars contain the virulence plasmids (44).

An important virulence factor located on a plasmid previously shown to be common among predominant nontyphoidal serovars of *Salmonella* spp. is the *spv* operon (45, 46), which contains five genes (*spvRABCD*). Gebreyes *et al.* (47) found that one of the most important genes in this operon, *spvA*, is associated with MDR and the present findings support the hypothesis that occurrence of virulence factor *spvA* within a strain exhibiting specific MDR phenotypes may make strains clinically more relevant (46, 47, 48, 49, 50). Carriage of *spv*A among MDR strains may increase the propensity of such strains to be of major veterinary relevance; this is a potential public health concern because transfer of this plasmid to susceptible isolates could render them more virulent and resistant to multiple antimicrobial agents.

It has been estimated that approximately 90% of all antimicrobial agents used for food animal production are administered at sub-therapeutic levels, as prophylactics or for growth promotion (51).

A limitation of the present study is that the number of isolates of each *Salmonella* serovar was very small. Thus, the study should be viewed as a first, exploratory step to identify serovars of salmonellae harboured by imported turkey poults, an issue of great public health significance.

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Table I

Virulence factor targets and primers, including nucleotide sequences, PCR conditions and references

Gene designation	Location on SPI/Gene function	Oligonucleotide sequences (5'-3')	PCR conditions [a]			Product size (bp)	References
			Denaturing	Annealing	Extension		
*inv*A	Type III secretion system apparatus SPI-1/Invasion of macrophages	gtg aaa tta tcg cca cgt tcg ggc aa tca tcg cac cgt caa agg aac g	94°C for 60 s	64°C for 30 s	72°C for 30 s [b]	284	Salehi *et al*. (24)
*avr*A	SPI-1/Controls *Salmonella*-induced inflammation	cct gta ttg ttg agc gtc tgg aga aga gct tcg ttg aat gtc c	95°C for 30 s	58°C for 30 s	72°C for 30 s [b]	422	Huehn *et al*. (25)
*ssa*Q	SPI-2/Secretion system apparatus protein, component of second T3SS	gaa tag cga atg aag agc gtc gtc c cat cgt gtt atc ctc tgt cag c	95°C for 30 s	58°C for 30 s	72°C for 30 s [b]	455	Huehn *et al*. (25)
*mgt*C	SPI-4/ Mg^{2+} uptake	tga cta tca atg ctc cag tga at att tac tgg ccg cta tgc tgt tg	95°C for 30 s	58°C for 30 s	72°C for 30 s [b]	677	Huehn *et al*. (25)
*sii*D (*Spi4*D)	Type I secretion SPI-4	gaa tag aag aca aag cga tca tc gct ttg ttc acg cct ttc atc	95°C for 30 s	58°C for 30 s	72°C for 30 s [b]	655	Hauser *et al*. (23)
*sop*B	SPI-5/Inositol polyphosphate phosphatase that promotes macropinocytosis, regulates SCV localisation, and promotes fluid secretion	tca gaa gRc gtc taa cca ctc tac cgt cct cat gca cac tc	95°C for 30 s	58°C for 30 s	72°C for 30 s [b]	517	Huehn *et al*. (25)
*gip*A	Gifsy-1 bacteriophage/Peyer's patch-specific virulence factor	acg act gag cag cgt gag ttg gaa atg gtg acg gta gac	95°C for 30 s	58°C for 30 s	72°C for 30 s [b]	518	Huehn *et al*. (25)
*sod*C1	Gifsy-2 bacteriophage/Periplasmic	cgg gca gtg ttg aca aat aaag	95°C for 30 s	58°C for 30 s	72°C for 30 s [b]	424	Huehn *et al*. (25)

	Cu, Zn-superoxide dismutases	tgt tgg aat tgt gga gtc					
*sop*E1	Cryptic bacteriophage/Promotes membrane ruffling and disrupts tight junctions	act cct tgc aca acc aaa tgc gga tgt ctt ctg cat ttc gcc acc	95°C for 30 s	58°C for 30 s	72°C for 30 s [(b)]	422	Huehn *et al*. (25)
*spv*C	pSLT /A phosphothreonine lyase required for complete virulence in murine models	acc aga gac att gcc ttc c ttc tga tcg ccg cta ttc g	95°C for 30 s	58°C for 30 s	72°C for 30 s [(b)]	467	Huehn *et al*. (25)
*bcf*C	Chromosome/Bovine colonisation factor, fimbrial usher	acc aga gac att gcc ttc c ttc tgc tcg ccg cta ttc g	95°C for 30 s	53°C for 30 s	72°C for 30 s [(b)]	467	Huehn *et al*. (25)

a) PCR for 35 cycles
b) After 30 cycles, final extension step of 4 min at 72°C

PCR: polymerase chain reaction

pSLT: *Salmonella* Typhimurium virulence plasmid

SCV: *Salmonella*-containing vacuole

SPI: *Salmonella* pathogenicity island

Table II

Distribution of resistance to antimicrobial agents among five *Salmonella enterica* serovars isolated from turkey poults in Egypt

Salmonellae were isolated from eight of 150 faecal samples. In one case, the strain could not be serotyped and only the seven typed isolates have been considered here

Antimicrobials	Distribution of resistance to antimicrobials				
	S. Enteritidis (2/7)	*S*. Typhimurium (2/7)	*S*. Larochelle (1/7)	*S*. Altona (1/7)	*S*. Virchow (1/7)
Penicillins					
Ampicillin	1/2	0/2	0/1	1/1	0/1
Amoxicillin	0/2	0/2	0/1	1/1	0/1
Aminoglycosides					
Gentamicin	0/2	0/2	0/1	1/1	0/1
Neomycin	0/2	1/2	1/1	1/1	0/1
Streptomycin	0/2	0/2	1/1	1/1	1/1
Fluoroquinolones					
Ciprofloxacin	0/2	0/2	0/1	0/1	0/1
Nalidixic acid	2/2	1/2	1/1	1/1	0/1
Norfloxacin	1/2	0/2	0/1	1/1	0/1
Phenicols					
Chloramphenicol	2/2	2/2	0/1	1/1	1/1
Polymyxin					
Colistin sulfate	0/2	0/2	0/1	0/1	0/1
Tetracyclines					
Tetracycline	2/2	1/2	0/1	1/1	0/1
Sulfonamides					
Trimethoprim	2/2	0/2	0/1	1/1	0/1
Trimethoprim–sulfamethoxazole	2/2	0/2	0/1	0/1	0/1

Table III
Distribution of virulence gene combinations and antibiotic resistance phenotypes in *Salmonella enterica* serovars isolated from imported and domestic turkey poults in Egypt
Bold indicates consistent occurrence of genes

Serovar	Origin	Virulence gene combinations	Antibiotic resistance
Enteritidis	Imported	***inv*A**, ***avr*A**, ***mgt*C**, ***ssa*Q**, ***sod*C1**, ***sop*B**, ***spv*C**, ***bcf*C**	Chl, Neo, Na, Tet, Tri, Sxt
Enteritidis	Imported	***inv*A**, ***avr*A**, ***mgt*C**, ***ssa*Q**, ***sod*C1**, ***sop*B**, ***spv*C**, ***bcf*C**	Amp, Chl, Na, Neo, Nor, Tet, Tri, Sxt
Typhimurium	Imported	***inv*A**, ***sod*C1**, ***sop*B**, ***spv*C**, ***bcf*C**	Chl
Virchow	Imported	***inv*A**, ***avr*A**, ***ssa*Q**, ***mgt*C**, ***sop*B**, ***bcf*C**	Chl, Str
Larochelle	Imported	***inv*A**, ***avr*A**, ***mgt*C**, ***sod*C1**, ***sop*E1**, ***sop*B**, ***bcf*C**	Na, Neo, Str
Non-typeable	Imported	***inv*A**, ***ssa*Q**, ***mgt*C**, ***sii*D**, ***sop*B**, ***bcf*C**	Chl, Neo, Nor
Typhimurium	Domestic	***inv*A**, ***sop*B**, ***bcf*C**	Chl, Na, Neo, Tet
Altona	Domestic	***inv*A**, ***avr*A**, ***ssa*Q**, ***mgt*C**, ***sii*D**, ***sop*B**, ***bcf*C**	Amo, Amp, Chl, Gen, Na, Neo, Nor, Str, Tet, Tri

Amo: amoxicillin
Amp: ampicillin
Chl: chloramphenicol
Gen: gentamicin
Na: nalidixic acid
Neo: neomycin
Nor: norfloxacin
Str: streptomycin
Sxt: trimethoprim-sulfamethoxazole
Tet: tetracycline
Tri: trimethoprim