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Antimicrobial Resistance and Production of Extended Spectrum Beta-Lactamases in *Enterobacteriaceae* from Birds in Bangladesh

BADRUL HASAN

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Abstract
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The dissemination of members of the *Enterobacteriaceae* family with extended spectrum beta-lactamases (ESBLs) and metallo-beta-lactamases (MBLs) has become a global concern. ESBLs and MBLs have been reported in humans, domestic animals, wildlife and the environment, and their isolation frequencies are increasing rapidly worldwide. Most studies have been performed in developed countries and quite few in developing countries, where the antibiotic consumption is often poorly controlled. To explore the environmental contamination of antibiotic resistance in Bangladesh, and of ESBLs and MBLs in particular, fecal samples from poultry and wild birds were studied in this thesis.

Samples were collected from both sick birds (poultry having *Escherichia coli* infections) and healthy birds (free-range poultry, seagulls and crows) residing in different environmental niches. Samples from patients and fresh/sea water were included, to follow the chain of antibiotic resistance in bacteria from humans to the environment. Information regarding the antibiotic usage in poultry production was also collected. The susceptibility of avian *E. coli* isolates cultured with and without selective pressure was tested against antibiotics commonly used in human and veterinary medicine in Bangladesh. Special attention was paid to ESBL-producing isolates, which were further characterized genetically.

The results of the studies showed that *E. coli* isolates from commercial poultry, free-range poultry, gulls and crows were resistant to several classes of antibiotics, and that the level and spectrum of antibiotic resistance varied between different bird populations. There was no NDM-producer found among the birds, but ESBL-producing *Enterobacteriaceae* could be found in up to 59% of the crows, the birds with the highest carriage rate of multiresistant *Enterobacteriaceae* of all bird species studied. The most common ESBL-type was CTX-M-15, which also is the most common in the human population in Bangladesh. Birds also shared clinically important sequence types with humans, including *E. coli* clone O25b-ST131.

In conclusion, ESBL-producing bacteria with multiresistance are easily spread to wild birds. Their opportunistic feeding behavior at poorly managed hospital waste dumps and nearby water bodies makes them into both reservoirs and active spreaders. The high level of antibiotic resistant and ESBL-producing bacteria in the bird population of Bangladesh is worrying, and there is no easy solution in sight. Nationwide programs are necessary to both improve the management of hospital waste and sewage and the control of the antibiotic usage to prevent further environmental contamination.

Keywords: Antimicrobial Resistance, ESBL, CTX-M, NDM, Poultry, Wild birds, E. coli, Bangladesh, Enterobacteriaceae, Antibiotics

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‘‘Seek knowledge from the cradle to the grave ’’

‘‘Knowledge is maintained only through teaching’’

Great Prophet Muhammad (saw)

To My Father

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Hasan B**, Faruque R, Drobni M, Waldenström J, Sadique A, Ahmed KU, Islam Z, Parvez MB, Olsen B, Alam M. High prevalence of antibiotic resistance in pathogenic *Escherichia coli* from large and small scale poultry farms in Bangladesh. Avian Diseases, 2011, 55(4):689-692.

II **Hasan B**, Sandegren L, Melhus Å, Drobni M, Hernandez J, Waldenström J, Alam M, Olsen B. Antimicrobial drug-resistant *E. coli* in wild birds and free-range poultry, Bangladesh. Emerging Infectious Diseases. 2012 Dec;18(12):2055-8.

III **Hasan B**, Drobni P, Drobni M, Alam M, Olsen B. Dissemination of NDM-1 .The Lancet Infectious Diseases, 2012, 12(2):99-100.

IV **Hasan B**, Melhus Å, Sandegren L**,** Alam M, Olsen B. The seagull (*Larus brunnicephalus*) as an environmental bio-indicator and reservoir for antibiotic resistance on the coastlines of the Bay of Bengal. (Manuscript)

V **Hasan B,** Melhus Å, Alam A, Akter l, Olsen B. Association between hospital waste and the house crow (*Corvus splendens*) in the dissemination of antibiotic Resistance and the epidemic *Escherichia coli* clone O25b-ST131 in Bangladesh. (Manuscript)

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Abbreviations

ATCC	American Type Culture Collection
CTX-M	Cefotaximase
CMCH	Chittagong Medical College Hospital
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESBL	Extended Spectrum beta-Lactamases
MDR	Multidrug resistant
MLST	Multilocus Sequence type
MBL	Metallo-beta-lactamase
NRI	Normalized Resistance Interpretation
NDM	New Delhi metallo-beta-lactamase
OIE	World Organization for Animal Health
PCR	Polymerase chain reaction
rep-PCR	repetitive element PCR
RMCH	Rajshahi Medical College Hospital
ST	Sequence Type
SHV	Sulfhydryl variable
TEM	Temoniera
WHO	World Health Organization

Introduction

Antibiotic resistance

The development of antimicrobial agents is one of the most significant achievements in the medical history. The word 'antibiotic' is derived from the Greek words, *anti-* (against) and *bios* (life), and it was used before it was established that infections were caused by bacteria, viruses, fungi or prions. Therefore, an antibiotic drug was considered to be a substance which could kill any living organism. However, the word today is used in medical and veterinary sciences to denote a substance, either a chemical agent or a product from a living organism, which kills or inhibits bacteria. Sulfa is–for example–a chemical agent, whereas penicillin is produced naturally by *Penicillium notatum.* Some antibiotic drugs are naturally derived products with chemical modifications, i.e. fluoroquinolones.

Since sulfa and penicillin were first introduced during the 1930s and 1940s, bacteria have revealed a remarkable ability to develop different types of resistance mechanisms which mediate resistance to antimicrobials that are, from the beginning, quite toxic to them. Antibiotic resistance is today one of the most serious threats to public health, and it is a global problem. The evolutionary consequence of a high selective pressure during the years is that there is no longer any antimicrobial drug for which resistance has not been documented.

Human medicine

Antimicrobial drugs have provided physicians with the ability to treat and prevent many infectious diseases, but, unfortunately, there has been a dramatic increase of antibiotic consumption with the growing size of the human population. Not all consumption is controlled by physicians. Non-prescription antimicrobial use has occurred worldwide, and outside of Northern Europe and North America it has been estimated to account for between 19% to 100% of the use (Morgan DJ, 2011).

Drug-resistant bacteria appeared initially in hospitals, where most antibiotics were being used (Levy SB, 1998). Penicillin-resistant *Staphylococcus aureus* appeared in London civilian hospitals very soon after the introduction of penicillin in the 1940s (Barber M, 1948). Similarly, *Mycobacterium tuberculosis* with resistance to streptomycin emerged in the community soon

after the discovery of this antibiotic drug (Crofton J, 1948). Multi-drug resistance was first detected among enteric bacteria, namely *Escherichia coli, Shigella and Salmonella* in the late 1950s to early 1960s (Watanabe T, 1963; Olarte J, 1983; Levy SB, 2001). Over the last two decades, MRSA (methicillin-resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant enterococci) have emerged worldwide as important causes of nosocomial infections (Top J, 2008). In the last few years, the frequency and spectrum of infections caused by antimicrobial resistant bacteria have increased in both hospitals and the community, and scientists have predicted that multi-drug resistance will override single-drug resistance in the present decade.

With the passage of time, different antibiotic resistance mechanisms connected with different classes of antibiotics have emerged and become combined. The problem with multi-resistant strains is twofold: they are usually very difficult to treat, and several classes of antibiotics will select them. Thus, they are extremely difficult to get rid of. The enzymes that drive this development are primarily different types of beta-lactamases, including extended spectrum beta-lactamases (ESBLs), *Klebsiella pneumoniae* carbapenemases (KPCs), and metallo-beta-lactamases (MBLs). Strains carrying these enzymes are clinically challenging and cost lives, particularly in developing countries.

Resistant strains can be traced from the community to the hospital and vice versa, indicating that drug resistance is no longer confined. Fuelled by increasing antimicrobial use, the frequency of resistance among different bacteria has escalated, especially in developing countries where antimicrobials have been readily available without prescription. Resistance rates in almost all types of bacteria are much higher in developing than in developed countries (Collignon P, 2012).

Resistant bacteria move readily from person to person, from hospital to hospital, and from country to country, showing that resistant bacteria recognize no geographic boundaries. The extent of the dissemination of antibiotic resistant bacteria is so wide, that even healthy humans in the remote Peruvian Amazon carry bacteria with resistance to the oldest antibiotics, i.e. ampicillin, tetracycline, sulfamethoxazole and streptomycin (Bartoloni A, 2009). The ultimate result of the spread of antibiotic resistance will be increased mortality, morbidity, prolonged hospital stays, higher medical costs and additional toxicity.

Veterinary medicine

The veterinary use of antibiotics is directed towards farm animals, pets, wildlife and animals raised in aquaculture. Apart from therapy and prophylaxis, antibiotics are consumed to increase growth and feed efficiencies. Data about the antibiotic consumption are not publicly available, rendering it difficult to determine the quantities consumed and for what purposes. However,

based on extrapolations and indirect methods, it has been estimated that 27.5 million pounds of antimicrobials are used for "non-therapeutic" purposes (growth promotion and disease prophylaxis), and another 2 million pounds are used for therapeutic purposes in animals (Mellon M, 2001). In February of 2000, according to a survey of the members of the Animal Health Institute, 17.8 million pounds of antimicrobials were used in animal production in 1998; 14.7 million pounds (83%) for prevention and treatment of disease, and 3.1 million pounds (17%) for growth promotion (Animal Health Institute, 2000).

Nine classes of antibiotics are exclusively used in animals (Pagel SW, 2012), but several classes are commonly prescribed in both veterinary and human medicine: penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, macrolides, nitrofuranes, nitroimidazoles, sulphonamides, trimethoprim, polymyxins and quinolones (Prescott JF , 2000). However, concerns about the selection and dissemination of antibiotic resistance between animals and humans, have led to the concept of 'critically important human antimicrobial agents'. These agents should be reserved for human use.

The use of penicillins, tetracyclines, macroilides and aminoglycosides is pronounced in veterinary medicine, and it has been so for more than 50 years (Pagel SW, 2012). Bovine mastitis, pneumonia in calves, metritis in cows, and erysipelas in pigs are commonly treated with penicillins. Streptomycin has been useful in the treatment of mastitis, leptospirosis, pneumonia, intrauterine infections and dysentery in pigs. Tetracyclines have been an important and successful part in prophylaxis and therapeutics for several diseases like bovine pneumonia, footrot, metritis, mastitis in sheep and goats, scours in pigs and calves, and colibacillosis and pasteurelosis in poultry.

Several studies have shown that the highest frequency of antibiotic resistance and selection pressure occurs in calf, swine and poultry processing (Prescott JF , 2000). In human medicine, antibiotic treatment strategy is based on individual patient health status. In veterinary medicine, with the exception of companion animals and horses, much more attention is paid to the flock or herd of livestock. This is particularly true for production animals which are kept in groups on the basis of age or sex.

A survey published in 2005 by the OIE (World Organization for Animal Health) revealed that some countries have no regulations controlling the use of antimicrobial agents in livestock. However, the majority of the countries do have laws and regulations governing the approval and use of veterinary medicines (Valacher JF, 2006). Even in developed countries, such as the United States, many antibiotics are readily available to the livestock farmers without any prescription (Green AL, 2010). Without any doubt, the introduction and use of antimicrobial agents in veterinary medicine had and still has an enormous impact on the health and welfare of animal species.

Antibiotic resistance is a complex phenomenon, especially in veterinary medicine, because of the number of animal species, the diversity of rearing environments, the differences in the range of pathogenicity mechanisms and complex epidemiology (Acar JF, 2012). Although antimicrobial resistance is a concern for animal health, little is known about the magnitude of this problem. A wide variety of animal pathogens have been reported to be resistant against different antimicrobial compounds, e.g. *E. coli* of calves, pigs, and poultry; *Pasteurella multocida* and *Mannheimia (Pasteurella) haemolytica* from cattle, and *Actinobacillus pleuropneumonia* and *Streptococcus suis* from pigs (Cote S,1991 ;Lee MD, 2000 ; Noble WC, 1992). Long-term fecal shading of resistant bacteria can occur if the animals are treated with subtherapeutic doses of antimicrobials (Williams RD, 1978). Generally, the diversity and magnitude of resistance is highly variable among animal pathogens in different geographic areas (Wells SJ, 2001 ; Dargatz DA, 2000).

Antibiotic use in animals contributes to the selection and spread of resistant bacteria among animals, between herds and also between countries (McEwen SA, 2002). From animals, the resistant bacteria can be transmitted to humans through direct contact, via food, etc. (Levy SB, 1976 ;van den Bogaard 2001; Seong CN, 2004; Martin LR, 2013), and eventually, the environment and water supply can become contaminated (Chee-Sanford JC, 2001).

Aquaculture

Aquaculture has become a significant supplier of food worldwide, with shrimp and pangasius production becoming popular in many Asian countries. Aquaculture continues to be the fastest growing animal food producing sector, and it outpaces the population growth (FAO, 2009).

Tetracyclines, macrolides, quinolones, sulphonamides, nitrofurans, chloramphenicol, are often administered in aquaculture in many Southeast Asian countries to promote growth and to treat or prevent infectious diseases (Shariff M, 1992; Boonyaratpalin M, 1996). There is a well-established relationship between the usage of specific antimicrobials in aquaculture environments and increases in antimicrobial resistant bacteria in the aquatic environment (Kerry J, 1994 ;Tendencia and Peña, 2001). The use of antibiotics in aquaculture may contribute directly to an increase in the level of antimicrobial resistance among bacteria in the fish intestines, in the aquatic environment (Austin and Al-Zahrani, 1988; DePaola A, 1995) and in human pathogens (Furushita M, 2003 ; Rhodes G, 2000). There is also an indirect route. Animals, often chickens and pigs, are kept in cages over or adjacent to fish ponds. The animal manure is thereby excreted into the fish ponds, where a release of inorganic nutrients supports the growth of photosynthetic organisms. The fish in turn, eat these. Chickens and pigs can carry resistant bacteria, including those that produce ESBLs. A high resistance frequency can, in

this manner, be maintained and added up from the livestock to the fish and aquaculture. Consequently, aquaculture production can act as a reservoir for multi-resistant bacteria and facilitate the dissemination of the resistance genes.

Environment and different ecological niches

Based on scientific evidence, it has been postulated that environmental organisms can act as reservoirs of antibiotic resistance genes. Antibiotic resistance is natural, ancient, and hard wired in the microbial pan-genome. However, the role of anthropogenic use of antibiotics in the emergence of resistant genes remains controversial. Samples from the Lechuguilla Cave in New Mexico, which had been isolated for over 4 million years, had surface microbes with highly resistant traits to antibiotics; some strains were resistant to 14 different commercially available antibiotics, including daptomycin, an antibiotic of last resort in the treatment of drug resistant Gram-positive pathogens (Bhullar K, 2012). There have also been reports of antibiotic resistance in microorganisms isolated from extreme natural habitats, including the deep terrestrial subsurface (Brown MG, 2009) and the deep ocean (Toth M, 2010), environments where human influence would seem largely absent.

Commensals in different wild species and environmental bacteria could be the hidden reservoirs of antibiotic resistance. An investigation in the UK found that wild rodents in England were potential carriers of multi-resistant bacteria with resistant phenotypes to human and veterinary antimicrobials (Gilliver M, 1999). A Finnish study mentions wild mammals, including moose and white-tailed deer, as carriers of *Enterobactericeae* resistant to cefuroxime and to streptomycin (Osterblad M, 2001). Wild birds, including long and short distance migratory species occupying different ecological niches and adapted with different feeding behaviors, can also harbor multi-drug resistant bacteria (Bonnedahl J, 2009; Hernandez J, 2010). Furthermore, antibiotic resistant *E. coli* has been isolated from ducks and geese (Tsubokura M, 1995; Fallacara DM, 2001; Cole, D 2005), cormorants (Dobbin G, 2005), birds of prey (Blanco G, 2007; Costa D, 2008), gulls (Camarda A, 2006; Dolejska M, 2007; Gionechetti F, 2008; Poeta P, 2008), feral pigeons (Radimersky T, 2010), passerines (Nakamura M, 1982; Dolejska M, 2008) crows ((Blanco G, 2009) rooks (Literak I, 2007).

Clinically relevant bacteria from the soil with resistance towards the most newly developed antibiotics have been found (D'Costa VM, 2006). Bacteria resistant to penicillins have been isolated from both treated and untreated wastewater in several studies (Martins da Costa P, 2006; Ferreira da Silva M, 2007). Cephalosporin-resistant bacteria have been isolated from natural water reserves such as surface water, soil and wetland sediment in different countries (Ash RJ, 2002; Esibu N, 2002; Halda-Alija L, 2004). There is also

a constant release of antibiotic resistant human and animal commensals and pathogens into the environment through wastewater and manure. This results in frequent and easy access to genes encoding antibiotic resistance in soil and aquatic environments (Kim and Aga, 2007; Kummerer K, 209).

Antibiotic resistant bacteria have been spread to more pristine areas with small human populations like those mainly restricted to research bases and very small-scale tourism, i.e. in wild and free range scavengers from different ecological niches in the Arctic and Antarctica. In 2008, a study on bacterial resistance in wild birds from the arctic tundra showed that there was resistance to all but three of the 17 antibiotic drugs tested (Sjölund M, 2008). Four years later, multi-drug resistant bacteria were found in penguins and nearby water bodies (Miller RV, 2009; Hernandez J, 2012).

This body of evidence supports the hypothesis that widespread resistance is a modern phenomenon linked to human and animal use of antibiotics, although the genetic background is ancient and embedded into the microbial pan-genome. To better understand how antibiotic resistance is spread, it is important to establish the presence of reservoirs. A reservoir is an ecological niche in which an infectious agent persists by a cycle of transmission, reproduction, or both (Tauxe RV, 1989).

Mechanisms of antibiotic resistance

Antibiotic targets

Bacteria have the unique ability to adapt themselves to various concentrations of a variety of toxic compounds and this capacity has been named "Antimicrobial Resistance." In order to appreciate the mechanisms of resistance, it is important to understand how antibiotics work. As a rule, these drugs act selectively on vital microbial functions with little to no effect on vertebrate host functions. The mechanism of action for antimicrobial agents can be categorized further based on the structural target or the function affected by the drug. These include inhibition of the cell wall synthesis, ribosome function, nucleic acid synthesis**,** folate metabolism or cell membrane function.

Resistance to beta-lactam antibiotics

The beta-lactam group of antibiotics is the largest and most commonly used group globally. It consists mainly of penicillins, cephalosporins, monobactams, carbapenems, and cephamycins, which are semi-synthetic compounds originating from fungi and bacteria (Walsh C, 2003). All drugs in the group possess a beta-lactam ring, the functional part of the antibiotic.

There are four major ways for a bacterium to develop resistance to beta-lactams: (1) production of beta-lactamases (enzymes that hydrolyse the beta-

lactam ring), rendering the antibiotic inactive, (2) altered penicillin-binding proteins (PBPs) with low affinity for beta-lactams, (3) lowered or lacking expression of porins in the outer membrane of Gram-negative bacteria, and (4) efflux (a pumping mechanism), which like (3) leads to difficulty for the beta-lactams to access the PBPs (Babic M, 2006).

Beta-lactamases are enzymes located in the periplasm on the outer surface of the inner membrane of the cell envelope in Gram-negative bacteria (Ghuysen JM, 1991). The *Enterobacteriaceae* family mainly produce beta-lactamases to deactivate beta-lactam antibiotics, and the first described resistance to β-lactams was the production of penicillinase by *E. coli* (Medeiros AA, 1997; Bush K, 2001; Bush K, 213). To date, over 1300 beta-lactamases have been reported (Bush K, 2013). The genes encoding them (*bla* genes) are located on either the bacterial chromosome, plasmids, transposons or integrons (Wright GD, 2005; Babic M, 2006; Drawz SM, 2010).

There are four classes of beta-lactamases (A–D) based upon DNA sequence similarity. Classes A, C, and D utilize serine on the active site, while the class-B enzymes (MBLs) usually utilize zinc. The updated classification system includes group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor-resistant beta-lactamases, ESBLs and serine carbapenemases; and group 3 (class B) MBLs.

Extended spectrum beta-lactamases

Extended spectrum beta-lactamases (ESBLs) are often acquired plasmid-mediated beta-lactamases that hydrolyze broader spectrum beta-lactams containing an oxyimino-group (e.g. ceftazidime, ceftriaxone, cefotaxime, aztreonam) as well as penicillins (Bradford PA, 2001; Paterson DL, 2005). They can be inhibited by clavulanic acid and tazobactam.

ESBLs have been found in the *Enterobactericeae* family, mostly *E. coli* and *K. pneumoniae*. Non-*Enterobactericeae* ESBL-producers are relatively rare, with *Pseudomonas aeruginosa* being the most important organism (Nordmann P, 1998; Poirel L, 2012). ESBLs have also been reported in *Acinetobacter spp., Burkholderia cepacia* and *Alcaligenes fecalis* (Bradford PA, 2001; Poirel L, 2012; Saha R, 2010).

There are several different types of ESBLs. The majority of the older ESBLs were derived from TEM-1 and SHV-1 (Bradford PA, 2001; Drawz SM, 2010; Bush K, 2013). TEM-1 was the first plasmid-mediated beta-lactamase in Gram-negatives. It was reported in 1965 from an *E. coli* isolate belonging to a patient in Athens, Greece, named Temoniera (hence the designation TEM) (Datta N, 1965). TEM-1 is able to hydrolyze penicillin and the early cephalosporins. It is the most commonly encountered beta-lactamase in Gram-negative bacteria. It has spread all over the world, and is now found in members of *Enterobacteriaceae*, *P. aeruginosa, Haemophilus*

influenzae and *Neisseria gonorrhoeae* (Livermore DM, 1995). More than 200 TEM-type enzymes have been described so far, and the majority of them are ESBL-producers (lahey.org/studies). The first plasmid-encoded ESBL was, however, a variant of SHV-1. It was found in a *Klebsiella pneumoniae* strain from Germany in 1983 (Jacoby GA, 2006).

One of the newer ESBL types is CTX-M (cefotaximase), and its name reflects the potent hydrolytic activity against cefotaxime (Bonnet R, 2004). It is the most widespread ESBL enzyme, and it is assumed to have its origin in *Kluyvera* spp (Bonnet R, 2004; Drawz SM, 2010). At present, the CTX-M family is comprised of about 141 enzymes, which can be divided into five groups based on their amino acid sequence identities (Bonnet R, 2004). There are several ways of CTX-M classification. R Bonnet classified those using Arabic numerals: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. On the other hand, JD Pitout classified using Roman numerals: CTX-M-I, CTX-M-II, CTX-M-III, CTX-M-IV and CTX-M-V. Group I includes CTX-M-1, -3, -10 to -12, -15 (UOE-1), -22, -23, -28, -29, and -30. Group II includes CTX-M-2, -4 to -7, and -20 and Toho-1. Group III includes CTX-M-8. Group IV includes CTX-M-9, -13, -14, -16 to -19, -21, and -27 and Toho-2. Finally group V includes CTX-M-25 and -26. Toho-1 and Toho-2 are beta-lactamases that are structurally related to CTX-M-type enzymes, and they have similar hydrolytic activity against cefotaxime (Asma M Al-Jasser, 2006).

Epidemiology of ESBLs of the CTX-M type in human medicine

The first nosocomial outbreak of CTX-M-1 was recorded in an intensive care unit of a hospital in Paris, France (Kitzis MD, 1988). During the following 10 years, the documentation of what must be described as an almost explosive global dissemination of CTX-Ms in human hospitals increased with high speed (Bernard HC, 1992; Gniadkowski M, 1998; Radice M, 2002; Canton and Coque, 2006). Although the majority of ESBL-producing isolates have been derived from humans staying at hospitals (Bradford PA, 2001; Bonnet R, 2004; Pitout JD, 2010), isolates producing CTX-M type enzymes can now easily be acquired in the community (Arpin C, 2005; Pitout JD, 2005; Wieler LH, 2011). The CTX-Ms have been extremely successful and are able to pass into almost any kind of setting (Bonnet R, 2004; Pitout and Laupland 2008; Mshana SE, 2009), while the prevalence of classical ESBL enzymes like TEM or SHV has decreased (Livermore DM, 2007).

CTX-M-9 was reported for the first time from humans in Spain at the turn of the century (Sabaté M, 2000). A nationwide study that year demonstrated how in CTX-M-9 (27.2%), followed by SHV-12 (23.9%) and CTX-M-14 (20.5%), had become the most prevalent ESBL in *E. coli* strains from patients in Spanish hospitals (Hernandez JR, 2005). In contrast, CTX-M-1 is

predominant in Italy (Brigante G, 2005) and CTX-M-2 in most South American countries, Japan and Israel (Bonnet R 2004; Ben-Ami R, 2006). CTX-M-15 is not confined to a few countries but has a global distribution (Romero L, 2005; Brigante G, 2005; Lavollay, M 2006; Lartigue MF, 2005; Boyd DA, 2004). A number of molecular epidemiological studies have shown that the sudden increase of CTX-M-15-producing *E. coli* worldwide can largely be explained by the spread of a single clone, namely O25b:H4-ST131 (Nicolas-Chanoine MH, 2008; Rogers BA, 2011).

ESBLs in veterinary medicine

The first ESBL-producing bacterium from an animal (a laboratory dog) was isolated 1988 in Japan, and it was an *E. coli* strain with FEC-1 (non-TEM, non-SHV with cefotaxime resistance) (Matsumoto Y, 1988). *E. coli* strains producing ESBL have since then been identified in a wide range of animals, including fish (Hasman H, 2005; Biohaz, 2011; Pomba C, 2009; Jiang HX, 2012; Brinas L, 2002).

ESBLs of the TEM, SHV and CTX-M types have frequently been demonstrated in the microbiota of food-producing animals (Smet A, 2010a; Agersø Y, 2012). Food-producing animals are the primary reservoirs of zoonotic pathogens, and the detection of ESBL-production among *E. coli* and *Salmonella* strains has increased drastically in the last few years due to increased production and export of food animals and poultry. There are several examples of how food products can take part in the ESBL dissemination; *Salmonella* v*irchow* with CTX-M-9 production was isolated some years ago in Denmark from quails imported from France (Aarestrup FM, 2005). TEM-52 has been reported from poultry and poultry products in the Netherlands, from rabbits and poultry in Spain, and from poultry, beef and pork in Denmark (Hasman H, 2005; Brinas L, 2005; Blanc V, 2006; Cloeckaert A, 2007).

Reports indicate that the CTX-M enzymes now probably predominate in veterinary medicine as well (Vo ATT, 2007; Carattoli A, 2008; O'Keefe A, 2010; Smet A, 2010b; Carneiro C, 2010; Aarestrup FM, 2005). An *E. coli* isolate producing CTX-M-1 was first reported in a healthy dog in Portugal (Costa D, 2004). CTX-M-14 and CTX-M-55 are among the most prevalent ESBL types in companion animals and poultry in Asia (30–33%) and, to a lesser extent, in cattle and pigs (Ewers C, 2012; Seiffert SN, 2013), whereas CTX-M-14 and CTX-M-79 have been described in farmed fish in China (Jiang HX, 2012). Many reports deal with commercial and backyard free-range poultry (Bortolaia V, 2011; Ho PL, 2011; Asai T, 2011; Wasyl D, 2011; Bortolaia V, 2011; Kola A, 2012; Geser N, 2012; Zheng H, 2012; Hiroi M, 2012). Interestingly, there is, hitherto, no specific ESBL type associated with animals. Although, *E. coli* producing CTX-M-15 is the most

common finding in humans, it is not yet so in animals (Poirel L, 2002; Meunier D, 2006).

ESBLs in the environment and wild life

ESBL-producing *E. coli* is often used to trace the evolution of multi-resistant bacteria in the environment and wildlife. Although it is not clear how ESBL-producing *E. coli* isolates make their way into different ecological niches, they appeared in the environment about two decades after the first outbreaks of ESBL-producing *E. coli* in human clinical settings (Kitzis MD, 1988; Bauernfeind A, 1989; Costa D, 2006). It is likely that this is a spill-over form of environmental pollution from human activities (Arpin C, 2005; Pitout JD, 2005; Martinez, 2009a), rather than the result of a misuse of antimicrobials in natural environments (O'Brien, 2002).

Most studies on ESBL-*E. coli* in wildlife have been performed in Europe, where livestock and human density is very high. Frequent interaction between wildlife and humans and their activities can therefore be assumed, resulting in a higher risk for wildlife of acquiring resistant bacteria (Allen HK, 2010). Not surprisingly, there is a strong correlation between the abundance of antibiotic resistant bacteria and the interaction of wildlife with human activities (Skurnik D, 2006). However, even in remote places or pristine areas, these interactions take place (Sjölund M, 2008; Hernandez J, 2010; Pinto L, 2010), underlining the ease with which ESBL-producing *E. coli* isolates can be spread if no preventive measures are taken. It ought to be easy for humans to take care of their waste, especially considering how much humans influence various ecological niches of the planet via their feces, but for some reasons it is not.

A large number of wild animal species have been documented shedders of ESBL-producing *E. coli*, but most of them have been birds or rodents (Guenther S, 2011 review). The bird reports have covered everything from waterfowl to birds of prey (Poeta P, 2008; Bonnedahl J, 2010; Dolejska M, 2009; Hernandez J, 2010; Literak I, 2007, 2010; Simoes et al., 2010; Garmyn A, 2011; Wallensten A, 2011; Silva N, 2011; Costa D, 2006; Pinto L, 2010) and most of the common human-associated CTX-M types.

Although the detection of multi-drug resistant pathogens in wildlife is considered to be a new phenomenon, it could have been anticipated, as resistant bacteria other than intrinsically resistant soil organisms were found in environmental samples apparently free from any antimicrobial pressure decades ago (Sato G, 1978; Kanai H, 1981; Hughes VM, 1983; Tsubokura M, 1995). Thus, it can be concluded that antibiotic resistance in the environment is ancient and ubiquitous, but the addition of human activities has accelerated the development and made it even more dangerous.

Metallo-beta-lactamases

Metallo-beta-lactamases (MBLs) are a unique group of beta-lactamases. They differ structurally from the other beta-lactamases by their requirement for a zinc ion at the active site. They are inhibited by metal ion chelators, such as EDTA and dipicolinic acid (Laraki N, 1999; Marchiaro P, 2008), but they are resistant to clavulanic acid, tazobactam and sulphones. Functionally, they were once distinguished primarily by their ability to hydrolyze carbapenems. Today, there are some serine beta-lactamases that have acquired that ability, too.

According to the structural classification of β-lactamases, MBLs belong to class B (Bush K, 2010). This class is further divided into subclasses B1, B2, and B3, of which the class-B1 enzymes have emerged as the most clinically significant and are characterized by having two active-site zinc ions (Wang Z, 1999; Bebrone C, 2007).

MBLs are encoded either by genes that are part of the chromosomal framework (resident MBLs, for example in *Stenotrophomonas maltophilia*), or by heterologous genes acquired by horizontal gene transfer (acquired MBLs) (Cornaglia G, 2011). At least nine different types of acquired MBLs have been detected in members of the *Enterobacteriaceae* family, *P. aeruginosa, Acinetobacter baumannii*, and other Gram-negative non-fermenters (Cornaglia G, 2011). The most important enzymes clinically and for epidemiological dissemination are those types belonging to IMP, VIM, SPM, and NDM.

NDM is a novel type of MBL, which is most closely related to VIM-1/2 (32% identity). It was named after the city where it was first isolated (New Delhi, India) (Yong D, 2009). NDM enzymes belong to class-B1 and confer resistance to all beta-lactam antibiotics known (Kumarasamy KK, 2010). They are usually located on a readily transferable plasmid, which facilitates its transmission and intercontinental dissemination (Rolain JM, 2010).

NDM-1 was first detected in a strain of *K. pneumoniae* isolated in 2008 from a patient returning to Sweden from a visit in India (Kumarasamy KK, 2010). A variant of NDM-1 (designated NDM-2) which differed by a single amino acid was reported in 2011 (Kaase M, 2011). A series of further variants (NDM-3–NDM-8) has thereafter been reported on the Lahey Clinic beta-lactamase website (http://www.lahey.org/Studies/). The rapidity of the dissemination and development of new variants is illustrated by the fact that NDM-4 has recently been described in isolates recovered from patients previously hospitalized not only in India but also in Cameroon (Nordmann P, 2012; Dortet L, 2012).

NDMs are no longer rarities in India, Bangladesh or Pakistan (Islam MA, 2012; Day KM, 2013), and cases have been reported from a range of countries, including Oman (Poirel L, 2011), France (Poirel L, 2010), Spain (Struelens MJ, 2010) and Norway (Samuelsen O, 2011). Apart from environmen-

tal dissemination of NDM-1 in India (Walsh TR, 2011) and Vietnam (Isozumi R, 2012), reports concerning NDM-1 in livestock or poultry are limited. In 2010, a NDM-1-producing *Acinetobacter lwoffii* was recovered from chicken in China (Wang Y, 2012). Despite the low number of reports, the situation is worrying. This is due to the extreme treatment difficulties associated with these strains and to the fact that the strains and their plasmids may easily spread to a vast number of people since they emerged in areas with the world's largest human populations.

More avian aspects on bacteria and their resistance

E. coli in birds

E. coli resides in the intestinal tract of all mammals and in poultry and wild birds. The relative abundance of *E. coli* in avian species is dependent on climate, diet, body mass and host factors; hosts living in close proximity to human habitations are more likely to harbor *E. coli* than hosts living away from humans (Godon DM, 2003).

Pathogenic serogroups of *E. coli* may cause air sacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis, cellulitis or yolk sac infection in avian species. Any localized or systemic infection caused by *E coli* in birds is called colibacillosis (Barnes and Gross,2003). Collectively, these infections are responsible for significant economic losses in the poultry industry. For example, 43% of broiler carcasses were rejected because of gross lesions due to *E. coli* infection (Yogaratnam V, 1995).

Pathogenic coliforms are more frequent in the gut of newly hatched chicks than in eggs from which they hatched (Harry and Hemsly, 1965; Barnes and Gross, 2003), suggesting rapid spread after hatching. *E. coli* can be found in litter and fecal matter, and the bacterium persists for long periods, particularly when the litter is dry (Harry, 1964; Barnes and Gross, 2003). Pathogenic serotypes can also be introduced into poultry flocks through contaminated well water (Nagi and Raggi, 1972).

Bird-human transmission of bacteria and antibiotic resistance

Gathered data indicate that antibiotic resistance in enteric bacteria (*E. coli*, *Salmonella* spp, *Campylobacter* spp., and *Enterococci* spp.) can be transferred from birds to humans via the food chain or by direct contact. For instance, colonization of the intestinal tract with resistant *E. coli* from chickens has been observed in human volunteers (Linton A, 1977; Ojeniyi AA, 1989). Poultry has also been described as a source of antibiotic resistance in humans in northern India (Singh M, 1992). Occupational exposure to antimicrobial resistant *E. coli* from live bird contact in the broiler chicken industry

could be another important route of entry for antimicrobial-resistant *E. coli* into the human population (Ojeniyi AA, 1989; Levy SB, 1976; Price LB, 2007). Several studies from different geographic locations, i.e. Nigeria, India, Saudi Arabia, and Morocco, have described how *E. coli* from poultry with resistance to streptomycin, sulfonamides and tetracycline have ended up in poultry attendants or farmers (Ojeniyi AA,1985; Ojeniyi AA, 1989; Singh M,1992; Amara A, 1995). There was evidence of human exposure to 3rd generation cephalosporin resistant *E. coli* through consumption of broiler meat in Belgium (Depoorter P, 2012). Consumption of turkey meat containing vancomycin-resistant *Enterococcus faecalis*(VREF*)* was also considered as source of VREF in healthy humans in Denmark (Agersø Y,2008)
The knowledge of how human and avian gut flora is established and changed over time is limited and definitely incomplete. Furthermore, it is unclear how long the resistance determinants survive in the gut in different species, especially when considering how different the feeding behaviors can be, including the consumption of antibiotics. A way to follow the transmission of *E. coli* strains between species and explore the genetic structure of *E. coli* populations in birds and humans is to perform multi-locus sequence typing (MLST).

MLST is a method based on sequencing a small set of marker genes, usually only seven. So far, MLST analyses have revealed a zoonotic potential for most *E. coli* strains (Wirth T, 2006). Various ESBL-producing *E. coli* with sequence types associated with humans have, in addition, been found in wild birds and poultry in different countries (Bonnedahl J, 2010; Doi Y, 2010; Guenther S, 2010; Zong and Yu 2010), indicating an interspecies transmission. A typical example of a successful interspecies traveler is the epidemic and global *E. coli* clone O25b:H4-ST131. It has been found in humans (Nicolas-Chanoine MH, 2008), farm chickens (Overdevest I, 2011) and in a Glaucous-winged Gull in Kamchatka (Hernandez J, 2010).

Role of migration in the dissemination of resistant bacteria

Avian mobility and migration are remarkable biological phenomena, but they may also be epizootiologic factors. During the yearly migrations, birds have the potential of dispersing microorganisms that can pose a threat to the health of both the animals and the public over enormous geographical areas (Reed KD, 2003; Hubalek Z, 2004). Transmission between avian species can occur at breeding, during wintering, and at stop-over places where numerous birds of various species are concentrated. Thus, birds act as both reservoirs and vectors. They may also act as bio-indicators of the level of contamination of bacterial pathogens and genetic determinants of antibiotic resistance in the environment (Cole D, 2005; Bonnedhal J, 2010; Silva N, 2011; Radhouani H, 2012).

Some birds have an aquatic lifestyle and a highly mobile behavior. As a consequence, waterfowl are prone to be exposed to a diverse array of resistant pathogenic bacteria from several sources, including those originating from human sewage and agricultural run-offs. Due to their diverse presence in different in ecological niches and their ease in picking up human and environmental bacteria, wild birds might act as mirrors of human activities. They reflect the antibiotic resistance situation in a broader way than many other animals, and can thereby give an early warning.

Antibiotic resistance in developing countries

The World Health Organization (WHO) has estimated that 3.4 million people, mostly children, die every year from water-related diseases (mainly diarrhea). The majority of them live in 49 developing countries, which, according to the United Nations report, are experiencing a mounting number of cases of cholera and dysentery (Pandey S, 2006). The situation is further complicated by the emergence of bacteria with antibiotic resistance and production of ESBLs (Sosa AJ, 2010). In some cases, the bacterial strains are resistant to all available antimicrobial agents (Russo TA, 2003).

In developed nations, steps have already been taken to address this problem; however, not much has been done (WHO report on infectious diseases 2000; Shears P, 2000; Rahal 1997). Bacterial infections caused by antibiotic resistant strains are the main contributors to human and animal diseases in developing countries (Shears P, 2000; Shears P, 2001). Multi-resistant bacteria are more difficult to treat and require drugs that are often less readily available, more expensive and more toxic (Howard D, 2001).

Antimicrobial resistance is not only a medical problem but also an economic problem (Paladino JA, 2002; Cosgrove SE, 2003). Most developing countries have inadequate health care systems due to limited resources and financial capacity. The use as well as misuse of antimicrobials in humans, animals and plants affects the resistance problem. It is further aggravated by other socio-economic factors connected with misuse of antibiotics: education, knowledge, expectations, prescriber-patient interactions, economic incentives, characteristics of the health systems, the regulatory environment and the availability of resources (Byarugaba DK, 2004). Some common practices also contribute, such as self-medication, non-compliance, misinformation, misconceptions and false beliefs, pressure from ads and treatment expectations (Parimi N, 2002; Lipsitch M, 2002). However, the most important underlying factor in developing countries is poverty (Hossain MM, 1982).

Poverty leads to poor sanitation, immunodeficiency and malnutrition due to starvation, poor access to drugs and poor health care, all of which may propagate antimicrobial resistance. Poverty is, in addition, connected with

corruption. Corruption is a major factor in developing countries, and it cannot be ignored. In many developing countries, health care providers are influenced by financial gains and operate on a basis of profit. This may lead to unnecessary prescriptions (Byarugaba DK, 2004).

The situation in Bangladesh

The seventh most populated country in the world, Bangladesh, is a country with 58 trans-boundary rivers that empty into the Bay of Bengal. Most of the rivers are connected with wetlands called 'bills' and 'hawors.' Eighty percent of the population lives in villages, and these villages are riverside areas with many small hawors.

In most of the villages, the sanitation system is poor and, according to the Bangladesh Bureau of Statistics (BBS), about 89% of rural households rear poultry. In some cases, inhabitants in urban areas used to have small scale poultry farms. It is a backyard rearing system with very unhygienic conditions. Bangladesh hosts millions of birds wintering over from Europe, Siberia and the Himalayas. The co-existence of various bird species may increase the risk of exchanging pathogens between the domesticated birds and their wilder cousins or vice versa. Poultry farming has dramatically increased in recent years in Bangladesh, but infectious diseases constrain its development. Avian colibacillosis is the leading disease in birds of all ages, and its economical impact is great.

Fig 1. Direct interaction between wild birds and human

Fig 2. Direct interaction between human and poultry in rural and urban areas of Bangladesh

Fig 3. Domestic ducks searching for food close to a toilet

Fig 4. Natural water sources are the part of daily life for human and domestic birds.

The poor sanitation systems have not just affected the birds. Reports on the microbial quality of natural water reservoirs have shown that the water sources are unsafe for human consumption. *E. coli* with antibiotic resistance was one of the predominant findings (Alam M, 2006; Begum YA, 2007).

As in other developing countries in the world, the usage of antibiotics is frequent and is not closely monitored in Bangladesh. Between 50-90% of the drug purchases are made in the private sector without any prescription (Shamsuzzaman AKM, 2007). In a recent study, the most popular drug prescribed was ceftriaxone (30%) followed by cefixime (19%) (Fahad BM, 2010). Today, ceftriaxone is being recommended for 70% of human prescriptions with or without infections in Bangladesh (Kabir ML 2003). Costal aquaculture is an old tradition contributing to the national economy of Bangladesh, and 80% of hatchery owners use antibiotics without proper clinical indication. Chloramphenicol, erythromycin and tetracycline are the most common antibiotics used in aquaculture (Sheikh AU, 2006).

The consequence of this overuse and misuse of antibiotics is a high level of resistant bacteria in humans (Bhowmick BK and H Rashid, 2004), livestock (Akond MA, 2009, Biswas PK, 2001) and the environment (Alam M, 2006). In a poultry study from 2001 (Biswas PK, 2001), 100% of the *E. coli* isolates were already resistant to tetracycline and 20% to gentamicin. In another environmental study (Alam M, 2006) with *E. coli* from aquatic sources, the resistance frequencies were as follows: ampicillin 75%, tetracycline 65% and trimethoprim-sulpfamethoxazole 49%. Sixty-nine percent of the strains were resistant to more than four drugs and 24% were resistant to more than seven drugs.

ESBL-producing *E. coli* and *K. pneumoniae* with ESBL-production has become more common in clinical settings in Bangladesh, and the prevalence is high, at 43.2% for *E. coli* and 39.5% for *K. pneumoniae* (Rahman MM 2004). ESBL-producing *Shigella sp.* was reported during an outbreak in the capital city of Dhaka in 2001-2002 (Mahbubur R, 2004). Most *E. coli* and *Campylobacter* strains are totally resistant to fluoroquinolones, drugs that were once used as the last line treatment.

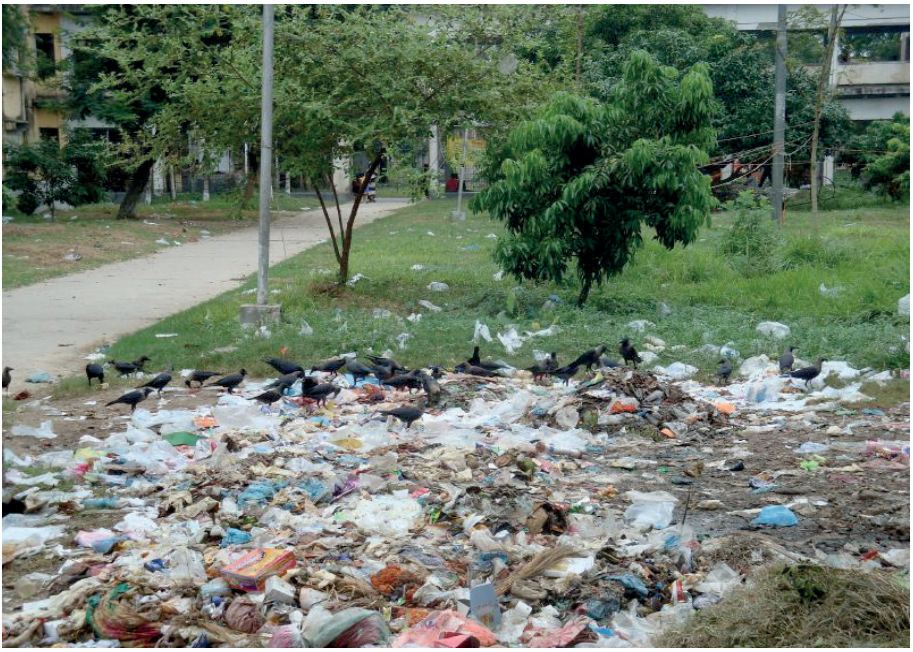

Fig 5. Crows searching for food in Hospital dumps in the ground of Rajshahi Medical College Hospital

Control measures

Control measures of hospital-acquired infection are poorly applied in Bangladeshi hospitals and clinics. There are no hospital waste management systems, and internal waste treatment facilities, such as incinerators, are rarely in place. Instead, the hospital waste is thrown around the hospital areas after careless permission from the hospital authorities. Solid hospital wastes are therefore often seen at the backyard of the hospital premises, and this area serves as the scavenging ground for stray dogs, gulls, crows, and drug users (Hassan M, 2008; Laskhmi K, 2003). Furthermore, the liquid wastes from hospitals flow through the sewage system into open ditches and the urban river systems, increasing an already high contamination load in the water environment.

Fig 6. 'Pharmacies', without any registered pharmacists, sell antibiotics without any prescription.

Monitoring of antibiotic resistance in Bangladesh now and in the future

There is no active or nationwide surveillance of antibiotic usage or antibiotic resistance among bacteria in Bangladesh. There are, however, several studies from research institutes and universities focusing on either the veterinary or the human side of the resistance problem. None have paid attention to the environment, possible reservoirs or the consequences of current practices. Furthermore, several methods have been used while surveying the antibiotic resistance, therefore the results are difficult to compare. Thus, it appeared important to use a more holistic approach and explore the contamination of antibiotic resistance in birds, water and humans. Furthermore, an internationally established method recommended by EUCAST was used, making it possible to compare results in the future.

This project was designed to answer some essential questions regarding the possible transfer of ESBL- and NDM-producing bacteria via the environment from urban and rural health care settings in a developing part of the world where health care facilities are poorly available. The project also focused on occupational hazards associated with poultry farming and health care. The role of hospital waste and antibiotic usage in the epidemiology and dissemination of multi-resistant bacteria appears to be an important area to explore and devote attention to in Bangladesh.

Aims

The overall aim was to study the current environmental contamination of antibiotic resistance among bacteria, and in *E. coli* in particular, due to use/overuse of antimicrobials in veterinary and human medicine and lack of infection control in Bangladesh.

Free-range domesticated birds and wild birds (ducks, crows and gulls) were used as indicators of the level of antibiotic resistance contamination in the environment.

The intermediate aims were the following:

I To determine the prevalence and diversity of antibiotic resistance in *E. coli* from different wild and domesticated birds;

II To determine and compare the prevalence and genotypes of extended spectrum beta-lactamases (ESBLs) in birds, humans and waters;

III To determine the presence of clonality and spread of certain clones of *E. coli* in wild bird populations with different migratory and feeding behaviours and humans;

IV To explore the impact of human hospital wastes, poultry farming, agricultural run-offs and wild birds in the dissemination of resistance determinants like CTX-M, TEM and NDM-1, in the environment.

Materials and methods

Sample collection

Collection of liver and heart samples (Study I)

In 2008 and 2009, commercial poultry farmers from the Dhaka and Gazipur districts of Bangladesh brought their dead and sick birds (Broiler, Layer) to the Gazipur Sadar Veterinary Hospital and Dhamrai Thana Veterinary Hospital. A total of 279 (67 in 2008 and 212 in 2009) sick or dead domestic fowl from different 260 poultry farms were subjected to postmortem examination during November 2008 to December 2009. Among these, 101 cases were diagnosed as avian colibacillosis. Diagnosis was made through postmortem examination after confirming the characteristic lesions of *E. coli* infection. Birds that displayed characteristics of perihepatitis and pericarditis were subjected to microbial examination. Heart and liver samples with lesions were transferred within 2 hours to the laboratory for further investigations using aseptic measures in a cold box with ice.

Collection of information on antibiotics usage (Study I)

During clinical investigations in the veterinary hospitals, the farmers were asked how they selected and used antibiotics at their farms: with or without prescriptions from veterinarians. They were also asked to name the antibiotics (commercial or generic) they chose for their farms or used before as growth promoting agents and against infections.

Collection of fecal samples (study II, III, IV and V)

Study II We performed sampling of wild birds, predominantly ducks, and domesticated chickens, ducks and geese in the city of Rajshahi, Bangladesh. A lake named 'Padmachar' located close to the city hosts several thousand wintering wild birds, and is also frequented by domesticated birds from surrounding farms. Samples (n=96; 41 wild ducks, 29 chickens, 23 ducks and 3 geese) were collected from the Padmachar area of Rajshahi district in January 2009. A cotton swab was swirled either in the cloaca or in bird droppings and was submerged in a bacterial freeze medium. Immediately after the sampling, the swabs were put into vials with a bacterial freeze medium

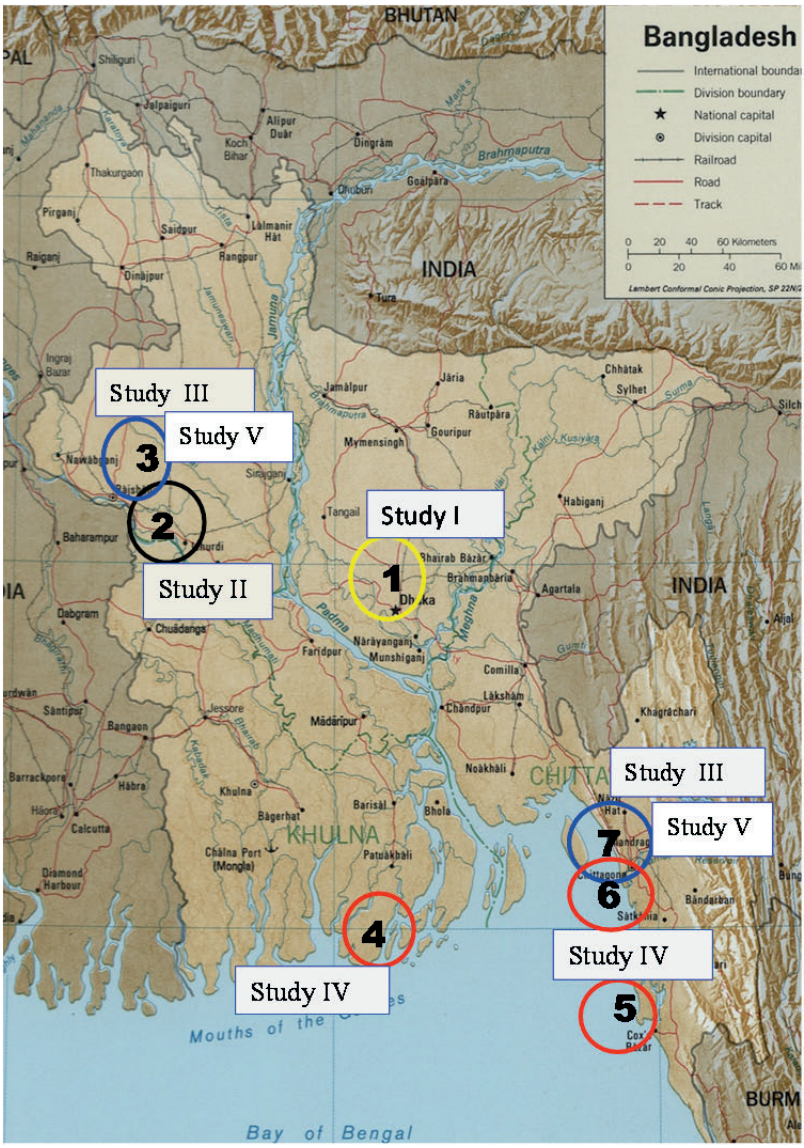

Fig 7: Geographical locations of our studies

1. Dhaka and Gazipur:Study I 2.Padmachar, Rajshahi: Study II

3. RMCH, Rajshahi: Study III and V 4.Kuakata beach, Patuakhali: Study IV

5 .Cox-bazar beach, Cox-bazar:StudyIV 6. Kornophuli River, Chittagong: Study IV

7. CMCH, Chittagong: StudyIII and V

(Lauria-Bertani broth; BD Sparks, USA, phosphate buffered saline containing 0.45% Na- citrate, 0.1% $MgSO_4$, 1% $(NH_4)_2 SO_4$ and 4.4% glycerol) and placed in a cool box with ice for later storage at -80^{o}C. The samples were shipped to Sweden on dry ice for analysis.

Study III and IV The opportunistic and adaptive feeder, the Brown-headed Gull (*Larus brunnicephalus*) migrates between the countries of Tajikistan, Southern China, Pakistan, India, Bangladesh, Sri-Lanka, Thailand and Hong Kong. Bangladesh is a wintering area for brown-headed gull along the coasts of the Bay of Bengal. Kuakata and Cox-Bazar on the shores of Bay of Bengal, are two popular tourist attractions in Bangladesh. Close to these beaches, thousands of gulls roost. During January 2010 and February 2010, 150 fecal samples from Brown-headed Gulls were collected from Kuakata (n=97) and Cox-Bazar (n= 53) beaches of Bangladesh. Fecal droppings were collected by sterile cotton swabs and handled as previously described. After sampling in the field in Bangladesh, all the samples were immediately stored in -80^{o}C and shipped to Sweden for analysis

Study III and V In this study, sampling was performed from the House Crow (*Corvus splendens),* a bird that lives and collects food from the inside areas of two major hospitals located in two different districts in Bangladesh. During 2010, 238 fecal samples were collected from crows on the grounds of Rajshahi Medical College Hospital (RMCH) and Chittagong Medical College Hospital (CMCH) in two different occasions. After samples were collected, they were stored and shipped to Sweden as described previously.

Collection of water samples (Study IV)

At the same time in 2010, 8 water samples were collected from the nearby sea and river which the gulls visited; 4 water samples from the Bay of Bengal at Cox-bazar beach area, and 4 samples from the Kornophuli River. Forty mL of sampled water was mixed with 10 mL of bacterial freeze media and immediately stored in liquid nitrogen tankers for initial storage in the field. Later all those samples were stored at -80^{o}C in a freezer. All samples were thereafter shipped to Sweden for analysis.

Collection of human samples (Study V)

During January 2012, we received 31 ESBL-producing clinical isolates (16 *K. pneumoniae*, and 15 *E. coli*) from patients from the Rajshahi Medical College Hospital and Chittagong Medical College Hospital. All these isolates were collected during the year 2011 from different human clinical cases in these hospitals on the occasions of routine microbiological investigation, either in the hospital or in the private diagnostic centers in Rajshahi and Chittagong districts.

Bacterial isolation and identification

In study I, heart or liver samples with characteristic lesions were deeply stabbed by swabs and cultured on 3% sheep blood agar (OXOID, Basingstoke, UK) and incubated overnight at 37ºC. Suspected *E. coli* colonies from blood agar were further inoculated onto MacConkey agar plates and confirmed by biochemical tests.

In studies II, III, IV and V, each fecal sample was plated onto a CLED (Lab M Ltd, Lancashire,UK and Uriselect 4 agar plate (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and presumed *E. coli* colonies were isolated and further characterized by biochemical testing and API 20E biochemical strips (bioMerieux SA, Marcy-l'Etoile, France) for species conformation.

In Study IV, the water samples were thawed, vortexed and filtered (35 mL) through Millipore filters (0.45 µM, SAS, Molsheim,France). The filters were placed on CLED and cultured overnight. *E. coli* was isolated and confirmed by biochemical testing. In study IV, human clinical isolates were re-confirmed at their species level by biochemical testing and API 20E biochemical strips.

All isolates from all 5 studies were stored at -80ºC in bacterial freeze media containing Lauria-Bertani broth (BD Sparks, USA), phosphate buffered saline and 4.4% glycerol.

Screening for ESBL-producing bacteria

In Study II, III, IV and V, each fecal sample was enriched in brain-heart infusion broth (BHI broth; Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with vancomycin (16µg/ml), ICN Biomedicals Inc. Aurora, OH, USA) for 18h at 37°C, and subsequently inoculated and cultured overnight at 37°C on chromID™ ESBL plates (bioMérieux, Marcy L´Etoile, France) according to the manufacturer's instructions. Colonies were isolated and species identity confirmed by biochemical testing. ESBL production was confirmed with cefpodoxime/ cefpodoxim-clavulanic acid double disk test (MAST Diagnostics, Bootle, UK). However in study I, *E. coli* isolates that showed resistance against more than 6 antibiotics (including β-lactam antibiotics) were cultured overnight at 37°C on chromID™ ESBL plates. A cefpodoxime/ cefpodoxime+clavulanic acid double disk test (MAST Diagnostics, Bootle, UK) was performed on these isolates to confirm the ESBL-production and *E. coli* ATCC 25922 was used for the quality control for this test.

Screening for carbapenemase-producing bacteria

In study II, screening for carbapenem resistant isolates was done by plating on Mueller-Hinton agar plates supplemented with 2µg/ml and 8µg/ml meropenem and incubated overnight at 37°C. In study IV, *E. coli* isolates from

water were tested against imipenem(10µg) and in study V, only the human isolates were tested against carbapenems (imipenem 10µg, meropenem 10µg) by disk diffusion on Mueller-Hinton agar according to the recommendation of the EUCAST and *E. coli* ATCC 25922 was used as a quality control strain.

Antimicrobial susceptibility testing

*E. coli i*solates, with and without ESBL-selective pressure, were cultured on blood agar plate and incubated at 37º C overnight to achieve fresh cultures for antibiotic susceptibility testing. Each isolate was subjected to antibiotic testing by using a panel of the following antimicrobial agents that are common in human and veterinary medicine practice in Bangladesh: Depending on the study, *E. coli* isolates were tested against 11-15 different antibiotics in accordance with EUCAST: ampicillin (10µg), chloramphenicol (30µg), gentamicin (30µg), nalidixic acid (30µg), tetracycline (30µg), streptomycin (10µg), mecillinam (10µg), ciprofloxacin (5µg), cefuroxime (30µg), nitrofurantoin (15µg), tigecycline (15µg), trimethoprim-sulphamethoxazole (25µg), fosfomycin (50 µg), cephradine (30µg) and cefadroxil (30µg). All compounds were provided by Oxoid Ltd (Basingstoke, Hampshire, England). Resistance to antibiotics was determined by disk diffusion on Mueller-Hinton agar according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion test guidelines. *E. coli* ATCC 25922 was used as the reference strain in all tests. Breakpoints for antibiotic resistance were determined according to EUCAST and the NRI(Normalized Resistance Interpretation) method. The theory and interpretation behind the NRI method has described by (Kronvall G, 2003)

Detection of ESBL-producing genes (CTX-M, TEM, SHV)

All of the ESBL-producing isolates tested for CTX-M ESBL genes. Bacterial DNA was prepared from overnight cultures by heating the bacterial suspension to 95°C for 10 min. followed by centrifugation. In study II, the presence of $bla_{\text{CTX-M}}$ genotypes in the samples was detected using a multiplex real-time PCR protocol (Birkett CI, 2007), displaying group designation (CTX-M-1/-2/-8 and -9) of $bla_{\text{CTX-M}}$ positive isolates. Positive isolates were sequenced, using the GenScript BacReady Multiplex PCR system (GenScript Corporation, Piscataway, NJ, USA) and previously described methods (Edelstein M, 2004; Klint M, 2006). Presence of bla_{TEM} and bla_{SHV} were detected using another set of previously described primers (Pitout JD

1998) and a SYBR®Green-based real-time PCR protocol (Bonnedahl J, 2010), followed by sequencing. Control strains were *E. coli* ATCC 25922 (SHV and TEM negative), *Klebsiella pnueumoniae* ATCC 700603 (SHV positive and TEM negative), *E. coli* UKNE7630 (TEM positive and SHV negative).

In study IV and V, the detection of the bla_{CTX-M} genotype was performed using a previously described protocol (Pitbout JD, 2004) which displaying the group designation (CTX-M-I/II/III/ and IV) of bla_{CTX-M}-positive isolates. Positive isolates were sequenced using specific primers CTX-M3F, CTX-M-2R, CTX-M914R and CTX-M-914F. Sequencing of CTX-M-I and CTX-M-IV groups was done according to the method described previously (Klint M, 2006) using specific primers (Pitout JD, 2004). Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). Control strains were *K. pneumoniae* U0503875 (CTX-M-I), C2 (CTX-M-II), C8(CTX-M-III) and C9(CTX-M-IV).

Detection of NDM-1 gene

In study III, for detection of NDM-1 in ESBL producing isolates, a qPCR was developed using the *K. pneumoniae* plasmid pKpANDM-1 sequence (GenBank:FN396876.1) (Yong D, 2009) and Primer3 software (Steve R , 2000) A forward primer (NDM1_123F: 5'-CGG CGT AGT GCT CAG TGT C-3') and reverse primer (NDM1_281R: 5'-GCC CGC TCA AGG TAT TTT AC-3') were designed and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used. Cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s. Primer concentrations were 500 nM for both primers.

Epidemiological typing by repetitive element PCR

To determine different epidemiologic clones by genetic profiling of the ESBL-producing *E. coli* (study II, IV and V) and *Klebsiella pneumoniae* (study 5) a rep-PCR was performed. Bacterial DNA was prepared from overnight cultures by heating the bacterial suspension to 95°C for 10 min. followed by centrifugation. PCR reaction mixture (final concentrations) 1x Taq PCR buffer, 1.9 mM $MgCl_2$, 50 µM dNTPs, 0.625 µM primer ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and 0.6U Taq polymerase (QIAGEN GmbH, Hilden, Germany) in a total reaction volume of 20 uL. Cycling parameters were as follows; 1 min at 94°C, 1 min at 36°C, and 2 min at 72 °C for 45 cycles followed by a final extension at 72°C for 5 min. The amplified products were visualized in a 1% agarose gel stained with ethidium bromide. The gel was photographed, and the DNA bands were analysed

visually. Isolates differing by one strong band or more were assigned to different genotypes. Isolates with identical strong band patterns but with an addition or a loss of a weak band were assigned subtype numbers.

Multi-locus sequence typing (MLST) of ESBL-producing isolates

ESBL-producing *E. coli* isolates *(study II, IV, and V)* with different CTX-M genotypes were characterized by multilocus sequence typing (MLST). Seven standard housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) were used, as described by Wirth and co-workers (Wirth T, 2006). Cycling parameters were as follows for all genes: 30 s at 95°C, 30 s at 60°C, and 1.5 min at 72°C for 30 cycles. PCR products were sequenced at Eurofins MWG Operon, Germany and allele profiles and sequence types were determined via the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli/#).

Detection of the 025b-ST131 clone

All of ESBL-producing *E. coli* isolates that were sequence type (ST) 131 were assayed for the O25b-ST131 clone (study II, IV and V). A PCR protocol was used to perform this assay (Clermont O, 2009). PCR reaction mixture (final concentrations); Hotstar Taq Mix 1000 U (QIAGEN GmbH, Hilden, Germany), O25pabBspe.F(5′-TCCAGCAGGTGCTGGATCGT-3′),O25pabBspe.R (5′-GCGAAATTTTTCGCCGTACTGT-3′) and in a total reaction volume of 23 uL. Cycling parameters were as follows; 30s at 94°C, 30s at 68°C, and 30s at 72 °C for 35 cycles followed by a final extension at 72°C for 5 min. The amplified products were visualized in a 1% agarose gel stained with ethidium bromide.

ESBL-plasmid conjugation

In Study II, ESBL-producing strains from each genotype were tested for transferability of the ESBL-plasmid by conjugation. *E. coli* DA11782 (mcrA, Δ(mrr-hsdRMS-mcrBC), ΔlacX74, deoR, recA1, araD139Δ(ara-leu)7697, galK, rpsL, endA1, nupG, rif^R) was used as the recipient. Equal amounts of donor and recipient-overnight-cultures in Luria-Bertani broth were mixed and incubated without shaking overnight at 37°C. Approximately 10^9 CFU of conjugation mixture was plated on selective plates containing 10 µg/ml cefotaxime, 100 µg/ml rifampin and 50 µg/ml of nalidixic acid. The plates were incubated overnight at 37°C.

Results

Study I

In this study we investigated the current situation of antibiotic usage and antibiotic resistance in veterinary medicine with a focus on poultry production. In addition, we investigated the presence of ESBLs in poultry pathogens. We found that 82% (215 out of 260) of the farmers chose their own antibiotics without prescriptions (Table 1). They used different classes of antibiotics regularly as growth promoting agents and for disease prevention (Table 1). *E. coli* was isolated from the organs of the diseased poultry showing clinical lesions. Of 101 *E. coli* isolates, 56 were resistant to one or several antimicrobial compounds, and the rest of the 45 isolates were found to be completely susceptible to the tested antibiotics. All in all, resistance to 12 of the 13 compounds was found in the resistant isolates. Of 56 resistant isolates, 19 isolates were resistant to a single antibiotic and 37 isolates showed resistance to several antibiotics. The prevalence and diversity of the antibiotic resistance phenotypes are presented in Tables 2 and 3. Interestingly, resistance to human antibiotics was present, albeit at low levels: nitrofurantoin (2%), cefuroxime (1%), cefadroxil (1%) and mecillinam (1%). None of the pathogenic *E. coli* isolates produced any ESBLs in this study.

Study II

This study investigated the frequency of antibiotic resistance and ESBL-producers in domestic and wild birds and also describes the transferability and potential inter species exchange of ESBL producing *E. coli*. Bacterial growth was found in 90 out of 96 samples. *E. coli* was isolated from 66 samples, yielding an isolation rate of 73.3% regardless of bird species. Fifty-three percent (35/66) of the isolates were resistant to one or more antibiotic compounds. The most common resistance was to tetracycline, followed by ampicillin, trimethoprim-sulphamethoxazole and nalidixic acid (Table 2). Interestingly, *E. coli* isolates from wild birds were resistant to cefadroxil, fosfomycin, gentamicin and cephradine. Multi-drug resistance was found in 22.7% (15/66) of the isolates, and 13.6% (9/66) of the isolates was resistant to 4-5 different classes of antibiotics.

Table 1.. Information about antibiotic usage by poultry farmers of Gazipur and Dhaka districts in Bangladesh.

No. of Farmers		Location of the farms (Name of the districts)	Background info of Antimicrobial compounds used Commercial name (Generic Name)	Prescription from Veterinarians: Yes/No
51	215	Gazipur and Dhaka	Renamycin Powder (oxytetracycline),	No
40		Gazipur and Dhaka	Ampistin (ampicillin + colistin)	
26		Gazipur and Dhaka	Anticoli-H (nalidixic acid + neomycine),	
21		Gazipur	Otetravet (oxytetracycline)	
21		Gazipur and Dhaka	Doxy-A-vet (doxycycline),	
18		Gazipur and Dhaka	Sulprim vet (sulfadiazine +trimethoprim)	
11		Dhaka	Cipro-A vet(ciprofloxacin),	
10		Gazipur and Dhaka	Gentamicin 5% powder (gentamicin)	
7		Gazipur	Salcomplus (ciprofloxacin+ enrofloxacin + colistin)	
6		Gazipur and Dhaka	Ciprosol-10 (ciprofloxacin), Furavet (furazolidone)	
4		Dhaka	Moxacil vet(amoxicillin), Docolis powder(doxycycline)	
14	45	Gazipur and Dhaka	Cipro-A vet(ciprofloxacin), cosomix plus(sulfachlorpyridizine + trimethoprim)	Yes
10		Gazipur	Anticoli-H (nalidixic acid+ neomycine), Anidox(doxycycline 15%)	
9		Gazipur	Otetravet (oxytetracycline), Amoxivet (amoxicilin)	
6		Gazipur and Dhaka	Flumivet (flumequine),	
3		Gazipur and Dhaka	Orciflox-10% (ciprofloxacin)	
2		Dhaka	Renamycin Powder (oxytetracycline),	
1		Gazipur	Enradin (enramycin hydrochloride)	

Table 2. Antibiotic resistance phenotypes in randomly isolated *E. coli* from different wild birds and poultry in Bangladesh.

Antibiotic	Commercial poultry (Gazipur,Dhaka) N =101	Free-range poultry (Rajshahi) N=52	Wild-ducks* (Rajshahi) N=14	Brown-headed gull (Kuakata) N=59	Brown-headed gull (Cox-bazar) N=26	House crow (RMCH, CMCH) N=161
Tetracycline	46 (45.5%)	24 (46.1%)	1 (7.1%)	-	-	84 (52.2%)
Trimethoprim-sulphamethoxazole	27 (26.7%)	17 (32.7%)	2 (14.3%)	10 (16.9%)	11 (42.3%)	70 (43.5%)
Ampicillin	26 (25.7%)	15 (28.8%)	4 (28.6%)	11 (18.6%)	14 (53.8%)	54 (33.5%)
Cephradine	-	0 (0%)	1 (7.1%)	-	-	-
Cefuroxime	1 (1%)	2 (3.8%)	1 (7.1%)	0 (0%)	2 (7.7%)	5 (3.1%)
Cefadroxil	1 (1%)	0 (0%)	1 (7.1%)	0 (0%)	2 (7.7%)	7 (4,3%)
Nalidixic acid	26 (25.7%)	11 (21.2%)	1 (7.1%)	9 (15.2%)	10 (38.5%)	64 (39.8%)
Ciprofloxacin	13 (12.9%)	3 (5.8%)	1 (7.1%)	5 (8.5%)	9 (34.6%)	29 (18%)
Streptomycin	21 (20.8%)	3 (5.8%)	1 (7.1%)	-	-	33 (20.5%)
Gentamicin	2 (2%)	0 (0%)	1 (7.1%)	0 (0%)	0 (0%)	0 (0%)
Fosfomycin	-	0 (0%)	1 (7.1%)	-	-	-
Chloramphenicol	9 (8.9%)	4 (7.7%)	0 (%)	2 (3.4%)	0 (0%)	7 (4.3%)
Nitrofurantoin	2 (2%)	1 (1.9%)	0 (%)	2 (3.4%)	1 (3.8%)	1 (1%)
Tigecycline	0 (0%)	0 (0%)	0 (%)	0 (0%)	0 (0%)	0 (0%)
Mecillinam	1 (1%)	0 (0%)	0 (%)	1 (1.7%)	0 (0%)	0 (0%)

'-' Not tested , **Anas crecca, Anas strepera, Aythya fuligula , Rhodonessa rufina,*

Table 3. Diversity of resistant phenotypes among the 56 antibiotic resistant *E coli* isolates recovered from poultry in Bangladesh.

Phenotypes of Antibiotic Resistance	Number of Isolates
TE	15
SXT	1
NA	2
AMP	1
NA-SXT	1
S-TE	6
NA-TE	1
TE-SXT	3
TE-AMP-SXT	1
NA-TE-SXT	1
NA-S-AMP-SXT	2
NA-AMP-C-CIP	2
S-TE-AMP-SXT	3
NA-TE-AMP-C	1
NA-TE-AMP-SXT	2
NA-C-SXT-CIP	1
NA-S-TE-AMP-SXT	2
NA-TE-AMP-C-SXT	1
NA-S-TE-AMP-SXT-CIP	1
NA-TE-AMP-C-F-CIP	1
NA-S-TE-AMP-SXT-CIP	2
NA-TE-AMP-C-SXT-CIP	1
NA-S-TE-AMP-CFR-SXT-CIP-CXM	1
NA-S-TE-AMP-C-F-SXT-CIP	1
NA-S-TE-AMP-C-SXT-CIP-CN	1
NA-S-TE-AMP-SXT-CIP-CN	1
NA-S-TE-AMP-MEL-SXT-CIP	1

NA; Nalidixic acid , S; Streptomycin, TE; Tetracycline, AMP; Ampicillin, C; Chloramphenicol, CFR; Cefadroxil, F; Nitrofurantoin, MEL; Mecillinam, SXT; Trimethoprim–Sulphamethoxazole, CIP; Ciprofloxacin, CN; Gentamicin , CXM; Cefuroxime.

Table4. ESBL-genotypes and Sequence types in *E. coli* from human, wild birds and poultry in Bangladesh.

Host	Place	ESBL-genotype	Sequence Type
Gull	Kuakata	CTX-M-15	ST1727
Gull	Kuakata	CTX-M-15	ST648
Gull	Kuakata	CTX-M-15	ST2687*
Gull	Kuakata	CTX-M-15	ST853
Gull	Kuakata	CTX-M-15	ST48 (ST48 complex)
Gull	Kuakata	CTX-M-15	ST2688*
Gull	Kuakata	CTX-M-15	ST2689*
Gull	Cox-bazar	CTX-M-15	ST349 (ST349 complex)
Gull	Cox-bazar	CTX-M-14 like	ST131
Gull	Cox-bazar	CTX-M-15	ST345 (ST345 complex)
Gull	Cox-bazar	CTX-M-15 like	ST10 (ST10 complex)
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST46 ST46complex
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST38 ST38complex
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST131 O25b
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST3482*
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST58 ST155complex
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST1664
Crow	Rajshahi Medical College Hospital	CTX-M-14like	ST405 ST405complex
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST3482*
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST58 ST155complex
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST3486*
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST3780*
Crow	Rajshahi Medical College Hospital	CTX-M-1	ST3781*
Crow	Rajshahi Medical College Hospital	CTX-M-14like	ST131 O25b
Crow	Rajshahi Medical College Hospital	CTX-M-14like	ST131 O25b
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST354 ST354complex
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST3487*
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST2141
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST3488*
Crow	Rajshahi Medical College Hospital	CTX-M-15	ST354 ST354complex
Crow	Chittagong Medical College Hospital	CTX-M-15	ST131 O25b
Crow	Chittagong Medical College Hospital	CTX-M-15	ST3135
Crow	Chittagong Medical College Hospital	CTX-M-15	ST1706
Crow	Chittagong Medical College Hospital	CTX-M-15	ST354 ST354complex
Crow	Chittagong Medical College Hospital	CTX-M-15	ST131 O25b

Crow	Chittagong Medical College Hospital	CTX-M-15	ST3782*
Crow	Chittagong Medical College Hospital	CTX-M-14like	ST131 O25b
Common Teal	Rajshahi	CTX-M-15	ST1408
Common Teal	Rajshahi	CTX-M-15	ST1408
Tufted Duck	Rajshahi	CTX-M-15	ST1312
Domestic duck	Rajshahi	CTX-M-15	ST2141
Domestic duck	Rajshahi	CTX-M-15	ST2690*
Domestic duck	Rajshahi	CTX-M-15	ST448 ST448 Complex
Domestic duck	Rajshahi	CTX-M-15	ST405 ST405 Complex
Domestic duck	Rajshahi	CTX-M-15	ST2691*
Domestic duck	Rajshahi	CTX-M-15	ST648
Domestic chicken	Rajshahi	CTX-M-15	ST206 ST206 Complex
Domestic chicken	Rajshahi	CTX-M-1	ST744
Domestic chicken	Rajshahi	CTX-M-15	ST648
Domestic chicken	Rajshahi	CTX-M-14 like	ST131
Domestic chicken	Rajshahi	CTX-M-15	ST2450
Domestic chicken	Rajshahi	CTX-M-15	ST2692*
Domestic chicken	Rajshahi	CTX-M-1	ST744
Domestic chicken	Rajshahi	CTX-M-15	ST2693*
Domestic chicken	Rajshahi	CTX-M-15	ST224
Human	Rajshahi Medical College Hospital	CTX-M-15	ST694
Human	Rajshahi Medical College Hospital	CTX-M-15	ST3493
Human	Rajshahi Medical College Hospital	CTX-M-15	ST2659
Human	Rajshahi Medical College Hospital	CTX-M-15	ST410 ST23complex
Human	Rajshahi Medical College Hospital	CTX-M-15	ST617 ST10complex
Human	Rajshahi Medical College Hospital	CTX-M-15	ST131 O25b
Human	Medical Diagnosis, Rajshahi	CTX-M-15	ST131 O25b
Human	Medical Diagnosis, Rajshahi	CTX-M-15	ST131 O25b
Human	Medical Diagnosis, Rajshahi	CTX-M-15	ST131 O25b
Human	Medical Diagnosis, Rajshahi	CTX-M-15	ST131 O25b
Human	Rajshahi Medical College Hospital	CTX-M-15	ST648
Human	Rajshahi Medical College Hospital	CTX-M-15	ST167 ST10complex
Human	Chittagong Medical College Hospital	CTX-M-15	ST38 ST38complex
Human	Chittagong Medical College Hospital	CTX-M-15	ST101 ST101complex

The screening for carbapenamase-producers yielded no isolates. The overall prevalence of ESBL-carriage among birds was 30% (27/90) with 36 ESBL-producing *E. coli* isolates. Thirty-four of them belonged to the CTX-M-1 group (2 $bla_{\text{CTX-M-1}}$ and 32 $bla_{\text{CTX-M-15}}$) and two to the CTX-M-9 group (both CTX-M-14-like). Combinations of $bla_{\text{CTX-M-15}}$ or $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-1}}$ were detected in 50% of the isolates. None of the isolates harbored genes encoding SHV.

The genetic fingerprints of the ESBL-producing *E. coli* isolates identified 15 different genotypes (A-O), of which 13 of 36 belonged to type A and genotype A had subtypes (A2,A3 and A4). Genotype A was found in both wild and domestic birds, indicating a sharing of clones. MLST analysis revealed 15 different sequence types (Table 4). Four isolates had new allele types or a new combination of allele types and were given novel sequence types (ST2690-ST2693). Conjugation was successful for 9/18 isolates, indicating the transferability of plasmids carrying ESBL genes.

Table 5. ESBL Genotypes in *Enterobacteriaceae* from house crow

Bacterial species	No of Isolates	ESBL genotype
E. coli	150	CTX-M-1, CTX-M-14like, CTX-M-15, CTX-M-55, CTX-M-79
Escherichia sp.	1	CTX-M-15
Klebsiella pneumoniae	17	CTX-M-15
Raoultella terrigena	14	CTX-M-15
Enterobacter cloacae	3	CTX-M-15
Citrobacter freundii	3	CTX-M-15
Proteus mirabilis	1	CTX-M-14like

Study III

The possible presence of NDM-1-producing *Enterobactericeae* from birds with short and long distance migratory behavior and frequent contact with human activities was investigated in this study. ESBL-producing *E. coli*, *Enterobacter cloacae*, *K. pneumoniae*, *Citrobacter freundii*, and *Raoultella terrigena* from the wild bird feces (gulls and crow) were isolated after ESBL selective pressure. More than 59% (n=238) of the crows and 27% (n=150) of the gulls were ESBL-positive phenotypically. All isolates were PCR-negative for the NDM-1 gene, showing that this resistance determinant had not yet reached our chosen bio-indicators in Bangladesh.

Table 6. Phenotypic diversity of the multidrug-resistant *E. coli* isolates from crows in Bangladesh. The bacteria were grown without selective pressure.

Multi-drug profile	No. of Isolates
AMP, TSU, TET	6
AMP, TSU, AMI	1
AMP, AMI, TET	1
AMP, TSU, QUI	1
TSU, TET, QUI	10
AMP, TET, QUI	3
AMP, CAM, QUI	1
AMP, CEP, QUI	1
TSU, CAM, QUI	1
AMP, CEP, TET	1
AMP, TSU, TET, QUI	4
TSU, AMI, TET, QUI	4
AMP, TSU, AMI, TET	7
TSU, TET, CAM, QUI	2
AMP, TSU, AMI, TET, QUI	10
AMP, TSU, AMI, TET, QUI	1
AMP, TSU, TET, CAM	1
AMP, TSU, TET, NIT, QUI	1
AMP, CEP, AMI, TET, QUI	1
AMP, CEP, TSU, AMI, TET	1
AMP, CEP, TSU, QUI, AMI, TET,	3
AMP, CAM, TSU, AMI, TET, QUI	2

AMP: ampicillin, CEP: cefadroxil, cefuroxime, TSU: trimethoprim–sulphamethoxazole, AMI: streptomycin, gentamicin, TET: tetracycline, CAM: chloramphenicol, NIT: nitrofurantoin, and QUI: nalidixic acid, ciprofloxacin.

Study IV

This study explored the rate of environmental contamination of antibiotic resistance in *E. coli* by various human activities in coastal areas of Bangladesh reflected in seagulls and waters. *E. coli* isolates, with and without ESBL selective pressure, from gulls and water samples were tested per sample basis to a panel of antibiotics and further characterized. In fact, the resistances were found against all classes of tested antibiotics. All in all, nearly half (42.3%; 36/85) of the *E. coli* isolates, without ESBL selective pressure, from gulls were found resistant to one or more antibiotics. The most widely spread resistance phenotypes were found against ampicillin (29.4%), trimethoprim-sulphamethoxazole (24.7%), nalidixic acid (22.4%) and ciprofloxacin(2.4%). Resistance to 3 or more different classes of antibiotics was considered as MDR. MDR was found in 10 isolates (11.8%; 10/85). There was

considerable difference in resistance between the two gull colonies (Table 2). Of the four *E. coli* isolates derived from water, two were resistant to ampicillin and trimethoprim-sulphamethoxazole. One of these two was, in addition, resistant to ciprofloxacin.

Carriage of ESBL-producing *E. coli* was relatively high (17.3%; 26/150) in the gulls, whereas no ESBL-producers were found in the water. Thirty-one ESBL-producing members of the *Enterobacteriaceae* family were obtained from 26 different samples with the selective isolation method. Of these were 29 *E. coli*. Twenty-eight *E. coli* isolates were confirmed to harbor $bla_{\text{CTX-M-15}}$ or CTX-M-15 like genes and only one *E. coli* isolate carried $bla_{\text{CTX-M-14}}$. The remaining two consisted of a CTX-M-15 producing *K. pneumoniae* isolate and a CTX-M-55/79 producing *Escherichia alberti* isolate. ESBL-producing isolates were completely resistant to the tested penicillins and cephalosporins. None of the ESBL-producing *E. coli* isolates were resistant to mecillinam, nitrofurantoin or tigecycline. Additional resistance was exhibited to nalidixic acid (*n*=11, 37.9%), trimethoprim-sulphamethoxazole (*n*=9, 31%), ciprofloxacin (*n*=9, 24.1%), chloramphenicol (*n*=2; 6.8%), and gentamicin (*n*=2, 6.8%).

The rep-PCR yielded six different genotypes (A, B, C, D, E, F).Nearly half (*n*=14) of the ESBL-producing *E. coli* isolates belonged to genotype A. Birds from the two beaches shared genotypes A and C. Both these genotypes had subtypes (A1, A2, A3, C1). Birds were carrying clinically relevant ST types such as ST10, ST48 and ST131 (Table 4).

Study V

In order to investigate the environmental contamination of antibiotic resistance in areas with poor waste management in Bangladesh, fecal samples from 238 house crows living in the surroundings of two major hospitals were screened for members of the *Enterobacteriaceae* family with extended spectrum beta-lactamase (ESBL)-production. These were compared with 31 ESBL-producing patient isolates, and the susceptibility of *E. coli* isolates was tested. Of the 238 crow samples, 141 (59.2%) harbored an ESBL-producing member of the *Enterobactericeae* family. The total number of ESBL-producing isolates was 198, and the species distribution was as follows: *E. coli* (*n*=154), *Klebsiella pneumoniae* (*n*=21), *Raoultella terrigena* (*n*=14), *Enterobacter cloacae* (*n*=4), *Citrobacter freundii* (*n*=3), *Escherichia* sp. (*n*=1), and *Proteus mirabilis* (*n*=1). The presence of CTX-M type genes were found in 189 (95.4%) of the ESBL-producing isolates. CTX-M group I enzymes predominated, and sequencing yielded four different CTX-M types: CTX-M-1, CTX-M-15, CTX-M-55 and CTX-M-79 (Table 5). Most common was CTX-M-15 (95.2%; 180/189). Seventy-two percent of the CTX-M-producing *E. coli* isolates were resistant to tetracycline. The corresponding

figures for trimethoprim-sulphamethoxazole and nalidixic acid were 57.3% and 56.6%, respectively. A relatively high frequency of resistance was also observed for ciprofloxacin (41.3%), streptomycin (34%), chloramphenicol (11.3%) and gentamicin (10.3%). Resistance at lower frequency was found for mecillinam (2%) and nitrofurantoin (3.3%). None of ESBL producing isolates was resistant to tigecycline. Of the 150 CTX-M-producing *E. coli* isolates, 100 (66.7%) of them were multi-drug resistant (range 3-7 antibiotic classes). All 31 human isolates carried CTX-M-15. The frequency of antibiotic resistance among the 15 *E. coli* isolates was the highest of all *E. coli* populations. Apart from the 100 % resistance to ampicillin and cephalosporins, the resistance rates were as follows: ciprofloxacin 93.3%, trimethoprim-sulphamethoxazole 60%, gentamicin 33.3%, chloramphenicol 26.6%, nitrofurantoin 13.3%, mecillinam 6.6%, and tigecycline 6.6%. None of the isolates were resistant to imipenem or meropenem, but all of them were multiresistant (range 3-8 classes).

Without selective pressure, 65.8% of the *E. coli* isolates from crows were resistant to one or more of 13 antibiotics (table 2), and 39.1% (63/161) were multiresistant. They displayed wide diversity of multidrug-resistant phenotypes (table 6)

The rep-PCR provided information about the genetic relationships among isolates, and both the *E. coli* and the *K. pneumoniae* isolates were genetically diverse. There were, however, some small *E. coli* clusters, in which both human and crow isolates were included. The largest *E. coli* cluster was, however, not recognized by this method. It was caused by the epidemic O25b-ST131 clone, and it was shared by patients (*n*=5) from the Rajshahi Medical College Hospital/Medical Diagnosis Center and crows (*n*=6) from both hospital areas. None of the genotypes generated by human *K. pneumoniae* isolates were identified among the crows, but crows from the two hospital areas shared *K. pneumoniae* genotypes. The different sequence types yielded by MLST are shown in Table 4. ST131 and ST38-ST38-complex were shared between humans and crows.

Discussion

Use, misuse and overuse of antibiotics in Bangladesh

In Bangladesh, antibiotics are easily available for use without prescription from physicians and veterinarians. Frequency of farmers or persons purchase antibiotics without prescription in our study is similar to the previous study on antibiotic usage in human (Shamsuzzaman AKM, 2007). Furthermore, 3rd generation cephalosporins have become common antibiotics in combination with other antibiotics in veterinary practice. Misuse of antibiotics has occurred so much over the span of decades that the third generation cephalosporins are common in clinical settings of Bangladesh (Kabir ML, 2003). Unfortunately, no data is available about the quantity of antibiotics used per year in Bangladesh. Even more, there is no exact data on how many pharmaceutical industries are currently available in Bangladesh. Pharmaceutical industries have been increasing in the recent years in Bangladesh due to high export demands. However, antibiotics are frequently used in veterinary practice and also in the poultry-dairy sectors without a prescription from authorized persons. In our study, we found that 82% farmers regularly used broad spectrum antibiotics as growth promoting agents as well as for disease prevention, especially tetracycline, ampicillin, nalidixic acid, gentamicin, ciprofloxacin and trimepthoprim. Moreover, there are more than 95 poultry feed companies in Bangladesh in our study area, incorporating flavomycin, zinc bacitracin, tetracycline, gentamicin, streptomycin and trimethoprim-sulfamethoxazole in their feed mixes (Unpublished data). In Bangladesh, poultry litter is used as fertilizer for crop production and in ponds for fish and shrimp production, which would encourage environmental contamination by resistant strains. We believe some factors, such as lack of knowledge about good farming practices, education, antibiotics and the overall easy availability of antibiotics are influencing the irrational use of antibiotics in Bangladesh. Direct evidence of over and misuse of antibiotics in human and veterinary medicine was the starting point of this project.

Antibiotic Resistance in Veterinary medicine

Aside from pets, humans get the greatest amount of antibiotics used for livestock or food producing animals on a daily basis. Additionally, several

human classes of antibiotics are used in veterinary medicine. Thus, it is obvious to see the antibiotic resistance in veterinary medicine. Except for some small-scale studies (Biswas PK,2001; Islam MT, 2004; Akond MA, 2009) there is no wide-scale study or information about antibiotic resistance in veterinary medicine focusing on poultry. That was the reason why we investigated antibiotic resistance in poultry pathogens and antibiotic usages by farmers located in areas where the poultry industries are based. In this study, pathogenic poultry strains were tested against antibiotics common in human and veterinary medicine. Additionally, some other human last line antibiotics like mecillinam, cefuroxime and cefadroxil, were also tested.

The pathogenic *E. coli* isolates from Bangladesh showed considerable resistance phenotypes against all classes of antibiotics tested. Widespread resistance was found against tetracycline, ampicillin and trimethoprim-sulphamethoxazole and nalidixic acid, which also are recognized common resistance phenotypes types in domesticated and wild animals in contact with human activities (Sorum and Sunde 2001; Valarcher JF, 2006; Green AL, 2010; Acar JF, 2012). Resistance to tetracycline, ampicillin, trimethoprim-sulphamethoxazole, nalidixic acid, streptomycin, chromaphenicol and gentamicin was lower in our study compared to similar datasets from Korea (Kim T, 2007), as well as to clinical (Bhowmick and Rashid, 2004), poultry (Biswas PK, 2001) and environmental (Alam M, 2006) isolates from Bangladesh. Thirty seven (36.6%) of the isolates showed multi-drug resistance. None of these isolates were resistant to tigecycline. Interestingly, resistance was present to antibiotics, though at low levels, to nitrofurantoin , mecillinam, cefuroxime and cefadroxil, and it is a matter of concern that poultry pathogens have become resistant against these broad spectrum human antibiotics even though they are not used in veterinary medicine practices in Bangladesh. Broad spectrums of antibiotics like mecillinam, cefuroxime and cefadroxil are only used in the hospitals of Bangladesh, but avian isolates were found to be resistant against these antibiotics. So, it can be concluded that animal or bird pathogens can carry *E. coli* with human resistance phenotypes and the dissemination of antibiotic resistance is widespread and frequent regardless of place (i.e. farms, hospitals and the environment). This finding led us to investigate bird populations that have free access to food in different environmental niches and have migratory behavior, either short or long distance.

General Antibiotic resistance in *E. coli* without selective pressure

In Study II, on free range poultry and wild duck, of the 15 tested antibiotic compounds, resistance was found in 53% of the isolates to all antibiotics

except mecillinam, and tigecycline, and about 23% (n= 66) of the isolates were multi-drug resistant. In Study IV on gulls, tests against 11 different antibiotics resulted in more than 42.3% resistant isolates and 11.8% were multidrug resistant. In Study V on crow, *E. coli* isolates were tested against 13 antibiotics and 65.8% (n=161) of the isolates were found resistant to one or more of the tested antibiotics. And phenotypes of multi-drug resistance were found in 62 (39.1%) of the isolates having resistance against 3 to 6 different classes of antibiotics. From all studies, it can be concluded that the carriage tendency and diversity of antibiotic resistant bacteria in fecal indicators was highest in crow compared to other bird species. And all these bird species were carrying human antibiotic resistant phenotypes. The predominant antibiotic resistant phenotypes of wild birds and free range poultry thereby appear to correlate with the antibiotic prescription patterns applied by veterinarians and physicians practicing medicine in Bangladesh. Notably here, these birds neither received antibiotic treatment nor were they influenced by antibiotics directly and they collect their food from different environmental niches. It is a matter of concern that human associated antibiotic resistant phenotypes are reflected in fecal indicators of free mover avian species.

In Study IV, we also noticed considerable differences in antibiotic resistant phenotypes in two different gull colonies located in two different areas. The study showed that the environmental contamination has already extended far on the coastlines of the Bay of Bengal: over 40% of the *E. coli* isolates from gulls were resistant to one or more of the tested compounds, and the number of MDRs passed the 10% level. Compared with other gull studies from the Czech Republic, France and Sweden (Dolejska M, 2009 ; Bonedahl J, 2009; Bonedahl J, 2010), the antibiotic resistance was at a higher level and more diverse. Contributing factors for high resistance level in different birds could be a high concentration of poultry industries, hospitals, shrimp hatcheries, and a high density of human populations.

ESBLs and MBLs in wild birds and Environment

The overall prevalence of ESBL-producers in different bird species was as follows; 30% (study II), 27% (study III), 59% (study III, V) and it seems the crows are the best ESBL carriers. ESBL- producing bacterial species diversity was highest in crows. Other than *E. coli*, several different bacterial species were found in gulls and crows, such as, *Escherichia* sp., *E. albertii, K. pneumoniae, Raoultella terrigena*, *Enterobacter cloacae, Proteus mirabilis* and *Citrobacter freundii.* In this study, we mainly focused on CTX-M enzymes in *E. coli*, however TEM was included only in study II. Different types of ESBL genes were found in birds: CTX-M-1, CTX-M-14 like, CTX-M-15, CTX-M-15like, CTX-M-55/79, and TEM-1. Combinations of CTX-M

and TEM were noticed in both wild birds and free range poultry. Several gull species, like the Black-headed Gulls (*Larus ridibundus*), Yellow-legged Gulls (*Larus michahellis*) and Glaucous-winged Gulls (*Larus glaucescens*) from different countries were reported to be carriers of globally endemic $bla_{\text{CTX-M-15}}$ genotype (Bonnedahl J, 2009; Bonnedahl J, 2010; Hernandez J, 2010). Still, the crows had a higher frequency of ESBL-producers than the poultry and gulls, indicating that the hospital waste probably contains not only high levels of antibiotic drugs but also multi-drug resistant Gram-negative bacteria. These bacteria can in turn be rapidly spread to any animal with an opportunistic feeding behavior. We found wild birds and free range poultry to be predominant carriers of the $bla_{\text{CTX-M-15}}$ genotype, which indicates that the dissemination of this CTX-M-15 ESBL type is widespread in the environment. We also found dominance of $bla_{\text{CTX-M-15}}$ genotypes in human samples regardless of bacterial species, indicating that the CTX-M-15 genotype is endemic in humans as well as the different environmental reservoirs in Bangladesh. CTX-M-14 and CTX-M-55 is among the most prevalent β-lactamase types in companion animals and poultry in Asia (30–33%), and to a lesser extent in cattle and pigs (Ewers C, 2012; Seiffert SN, 2013). It is interesting to note that, despite the use of different cephalosporins in veterinary medicine, there is no specific β-lactamase associated with animals and that only a few *E. coli* producers of the CTX-M-15 variant, which is one of the most prevalent in humans, have been identified in animals and poultry (Poirel L, 2002; Meunier D, 2006).

Carbapenem resistance was completely absent in human, water and birds. In a recent study, $bla_{\text{NDM-1}}$-containing bacteria were reported in a few clinical cases in India, Pakistan, and Bangladesh (Kumarasamy KK, 2010). In a matter of a few months, Walsh and colleagues (Walsh TR, 2011) had analyzed swabs from patients and samples of surface and supply waters collected from central New Delhi, India and reported $bla_{\text{NDM-1}}$-containing bacterial species belonging to the families *Enterobacteriaceae*, *Aeromonadaceae*, *Vibrionaceae* and *Pseudomonadaceae*. The emergence and rapid dissemination of $bla_{\text{NDM-1}}$ in a densely populated country like India is an extremely worrying scenario, since it suggests that $bla_{\text{NDM-1}}$ producers are being leaked from hospitals. We believe that poor management of the hospital environment and medical waste could be one of many causes of environmental dissemination of disease agents. South Asian countries take less care of medical waste than Western countries. For example, in Bangladesh and India, medical facilities rarely have internal waste treatment facilities such as incinerators in place; thus, solid hospital waste is often seen at the back yard of the hospital premises, inviting scavengers such as stray dogs, crows, gulls and even drug abusers. Furthermore, the liquid waste from hospitals flows with the sewage into open ditches and the urban river systems where birds such as ducks, crows, and gulls feed and can subsequently spread agents over wide areas. Wild animals and birds in particular, have been reported to be carriers and

spreaders of hospital-acquired multi-resistant bacteria (Bonnedahl J, 2009). Recently, NDM-1 producers have been reported in food animals in China (Wang Y, 2012). More dangerously in a recent event, NDM-1prodcuing bacteria have been in the rivers and lakes in Vietnam (Isozumi R, 2012), indicating the spread mechanism is faster than thought before. Even though we did not find any NDM-producers in birds in Bangladesh, however, the presence of NDM-1 in the environment is an indication of wide dissemination in India and Vietnam and this situation can be occurred in Bangladesh if there is lack of proper hospital waste management system and environment monitoring of antibiotic resistance.

There was also a frequency difference in antibiotic resistance when it came to the water samples: the fresh water was more polluted with resistant bacteria than the sea water. Considering how the human activities are concentrated to the rivers, and that the dilution capacity of the sea is much greater, this is not surprising. The result suggest, however, that wild birds are a better choice than both sea or fresh water, if there is an need for an early indicator of the environmental contamination of multiresistant bacteria.

Transfer of ESBL genes

The data on the transferability of ESBL-producing genes located in the plasmids of *E. coli* from Study II were interesting. ESBL genes are movable and can transfer genes to other bacteria. The very rapid dispersal of CTX-M ESBLs during the last decade is probably because of carriage on plasmids and this is the main force behind the pandemic spread. The CTX-M genes are very mobile and are present throughout the *E. coli* population. The increased frequency of detection of specific resistance determinants in the environment does not seem to be linked to the expansion of bacterial clones, but rather to plasmid-mediated horizontal gene transmission, promoting dissemination in specific areas. In light of this, it remains necessary to improve our understanding of the factors that contribute to the transmissibility of resistance determinants in natural environments.

Clonal dissemination and human associated bacteria in wild birds

In Study II, the PCR-based genotyping showed the diversity of the ESBL-producing *E. coli* isolates, and 15 different genotypes were recognized in the studied bird populations. Interestingly, wilds birds shared strains with free-range poultry, and some of the domestic ducks shared strains with chickens. We believe that this sharing of strains might be due to a common usage of

natural water resources, i.e. lakes, ponds and rivers by both wild and domestic species. This also shows with what ease *E. coli* can travel between species and cause infections. In study IV, the epidemiological typing of the ESBL-producing *E. coli* isolates showed that there was one dominating cluster, designated genotype A. This genotype together with another genotype (C), indicated that gulls may act not only as bio-indicators and reservoirs but also as vectors. In Study IV, both the *E. coli* and the *K. pneumoniae* isolates were genetically diverse. There were, however, some small *E. coli* clusters, in which both human and crow isolates were included. The largest *E. coli* cluster was, however, not recognized by this method. It was caused by the epidemic O25b-ST131 clone, and it was shared by patients from the Rajshahi Medical College Hospital/Medical Diagnosis Center and crows from both hospital areas. None of the genotypes generated by human *K. pneumoniae* isolates were identified among the crows, but crows from the two hospital areas shared *K. pneumoniae* genotypes.

By MLST analysis, we found different human associated sequence types (STs), common avian STs and new STs. We found several human associated sequence types: ST448, ST405, ST744, ST648 and ST131 in wild ducks and domestic birds. *E. coli* ST448 has previously been reported clinically in Nigeria (Aibinu I, 2012), whereas recent reports from UK have associated ST405 with the widespread distribution of CTX-M-15 and other ESBL enzymes in uropathogenic *E. coli* (Gibreel TM, 2012). ST648 harboring CTX-M-15 has been isolated from wild birds in Germany (Guenther S. 2010) and humans in Canada (Pierano G, 2012). Metallo-beta-lactamases of the NDM type have not been found in the environment of Bangladesh yet, but ST405 and ST648 are associated with NDM-1 producers on the Indian subcontinent (Mushtaq S, 2011). Gulls were carrying human associated *E. coli* sequence types like ST48, ST131 and ST10. *E. coli* ST10 clone was reported from urinary tract infection cases in several Chinese hospitals (Cao X, 2011) and ST48 clone was reported from clinical and poultry ESBL producers in Denmark (Jørgensen RL, 2010) and Netherlands (Leverstein-van Hall MA, 2011), respectively. Of the 26 sequence types found in humans and crows, the majority were O25b-ST131. This clone is widely distributed globally, and has been estimated to stand for about 20% of the expansion of ESBL-producing *E. coli* (Rogers BA, 2011). It was therefore not surprising to find O25b-ST131 among both patients and crows, but worrying that they could spread that easily with the crows. Apart from ST131, the crows were carrying three human associated sequence types, i.e. ST38 ST38-complex, ST46 ST46-complex and ST58 ST155-complex.

Risk factors for high ESBL in Bangladesh

Several risk factors have been identified in this project that are associated with wide-scale dissemination of antibiotic resistance and ESBL-producing bacteria in Bangladesh.

a) High population density with increasing number of livestock.
b) Unhygienic and polluted environments like natural water reserves due to human activities.
c) Misuse and overuse of antibiotics in human and veterinary medicine.
d) Increased number of poultry farms close to human habitation and illiteracy of farmers.
e) Birds easily accessing the nearest water reserves; hospital grounds that are severely polluted.
f) Insufficient sewage and drainage facilities in urban and rural areas.
g) Industrialization (Pharmaceuticals), trade and tourism in coastal areas.
h) Insufficient healthcare facilities and lack of hospital waste management.

Antibiotic Resistance in Bangladesh; Zoonosis or Anthropozoonosis?

The presence of human associated ESBL-producing bacteria in the environment is a clear indication of spread from humans (anthropozoonosis). Other than natural antibiotic resistance, most of the resistance to human antibiotics either emerged from the farm animals or in humans or the environment because of selective pressure due to irrational exposure to antibiotics or excess of antibiotics. Human activities can influence the animals and wildlife, making them more prone to carry human and animal associated bacteria as environmental reservoirs (Blanco G, 2007; Kim S, 2007; Allen HK, 2010; Bonnedahl J, 2010; Guenther S, 2011; Herandez J, 2012). Thus, antibiotic resistance in different environmental reservoirs is a clear indication of the potential for resistant organisms to cycle between humans and animals and vice-versa.

Conclusions:

I Antibiotic resistance is a serious problem in veterinary medicine and in the environment more so than ever before. Antibiotic resistance is a major problem in poultry industry in Bangladesh.

II Domesticated and wild birds can carry human associated antibiotic resistant bacteria and antibiotic resistance genes.

III Without any direct influence of antibiotics, birds can carry antibiotic resistance traits, including ESBL-producers: ESBL-producers have already spilled over into the environment.

IV CTX-M-15 is endemic in bacteria from bird populations in Bangladesh.

V Birds, regardless of species, can carry ESBL-producing bacteria of anthropogenic origin or zoonotic potential.

VI Bacteria, more specifically ESBL-producing *E. coli*, can cross the species barrier.

VII Influence of antibiotics in the environment has reached an extreme point that is reflected through considerable resistance levels in wild bird populations with varied feeding behaviors.

VIII Human activities can influence antibiotic resistance in different environmental niches including bird populations.

IX Birds may act as important environmental bio-indicators and vector of the antibiotic resistance situation.

Future Perspectives

To reduce the emergence and spread of antibiotic resistance in Bangladesh, over-the-counter sales of antibiotics should be controlled and veterinarians should authorize all antibiotics for animal and aquaculture use. Illegal and non-prescription based selling of antibiotics should be stopped in human medicine. Irrational use of antibiotics, including cephalosporins and carbapenems, must be stopped by the government in order to minimize antibiotic selective pressure in the environment and prevent outbreaks of emerging resistant pathogenic strains. This study shows high levels of antibiotic resistance pressure in the environment. Therefore, antibiotic residues and resistance genes circulating in the environment should be studied in different ecological niches. A national database should be established regarding the dispensing of antibiotics in different areas. Food producing animals and food supply chains should regularly be monitored to reduce food-borne antibiotic resistance and contamination of food by resistant bacteria. Also the food/environment interaction should be studied in connection with antibiotic resistance to stop the environmental contamination of food. Further investigation is required, to study how the presence of metals and biocides in the environment can influence antibiotic resistance in environmental bacteria. Pharmaceutical industries should be located far from human habitation sites and easy access to aquatic sources for the disposal of industrial wastes should be stopped. This study should not be restricted to birds, however, for extensive study is also required in other environmental niches, such as the water, sewage treatment plants, healthy humans, and especially the hospitals. Extensive studies are also required in clinical scenes to know more about the epidemiology of antibiotic resistance in human medicine. Our report and previous studies suggest the urgent need for regular screening and nationwide surveillance of antibiotic resistance and investigation of ESBL types in hospitals, livestock and the environment consecutively. The research infrastructure must be developed to strength the field of molecular and epidemiological studies in Bangladesh.

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