AMR in Fisheries Sector, Detection & Control

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Introduction

Living organisms that multiply frequently and spread rapidly are very tiny in nature and cannot be seen in naked eye are microorganisms. Majority of the organisms are existing as beneficial flora in each and every niche and contributing to the basic biogeochemical cycle of the life. However, some of the microbes do exists as pathogenic to either human or animals including the fish/shellfish. Examples are Bacteria (e.g., *Staphylococcus aureus, Streptococcus pneumoniae*), viruses (e.g., Measles, Mumps), fungi (e.g., *Candida albicans,*), parasites (Coccidia etc).

Any chemical or drug that normally kill or limit their growth are called antimicrobials. It may be produced from other microbes as natural or synthetics by chemical process. Due to the exposure of several chemicals or to environments, the microbes are continuously evolving and enabling them to efficiently adapt to new environments and it makes them harder to be eliminated from the particular environment. Examples of antibiotic penicillin and ciprofloxacin, whereas antimicrobial refers to all microbes *viz.*, bacteria, viruses, fungi, and parasites. Hence, antibiotic or antimicrobial resistance (AMR) denotes the ability of microbes to resist the effects of drugs, so that either their growth is not stopped or they are not killed or both.

Antimicrobial resistances is the ability of microbes to grow in the presence of antimicrobial substances. Major factors which potentiates the spread of antibiotic resistances are misuse or overuse of antibiotics with or without professional oversight, growth promoting substances in food producing animals, inadequate or inexistent programmes for infection prevention and control (IPC), poor-quality medicines, weak laboratory capacity, inadequate surveillance, insufficient regulation of the use of antimicrobial medicines. WHO has predicted AMR as a major threat to the public health and estimated that by 2050 many human beings succumb to death due to treatment failure in AMR Fig.1.

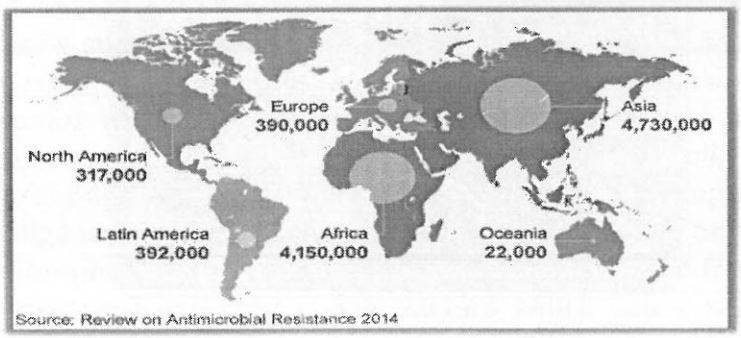

Fig.1. WHO report on death due to AMR by 2050.

Antibiotics are very critical compounds for preventing, controlling, and treating disease in human as well as farm animals. They act by preventing the essential metabolic process of bacteria which includes cell wall synthesis, DNA proliferation, and protein synthesis, antibiotics. However, the development of antibiotic resistance ie ineffectiveness of the antibiotics against bacterial pathogens makes situation critical than before. Multiple drug resistance is the resultant of the inadvertent or indiscriminate usage of antibiotics not only in the therapeutic both for human and animals but also for the prophylactic usage in the food producing animals including aquaculture. Steep increase in the antibiotic resistances have been reported in human as well as animals &and plants.

The relevance of detection and quantification of antimicrobial resistance or the genes responsible for the resistances are now shifted to bacteria from food producing animals or environment from clinically important pathogens. A decade back, clinically important and relevant organisms were *Enterobacteriaceae, Haemophilus* spp., *P. aeruginosaa, Neisseria gonorrhoeae, Staphylococcus* spp, *Streptococcus pneumoniae, Enterococcus* spp., *Streptococcus* spp., *Vibrio cholera, Helicobacter pylori and* potential agents of bioterrorism like *Bacillus anthracis, Yersinia pestis, Burkholderia mallei, and Burkholderia pseudomallei.* However, the current situation is more alarming due to the rapid emergence of antimicrobial resistances to newer pathogens and or evolution of more antibiotic resistances to already existing AMR pathogens. The condition is more alarming in bacteria from environment including aquatic environment. At this conjuncture, it is very important to understand the prevalence of antibiotic resistant pathogens in their defined geographical region inorder to control their rapid spread. Detection or determination of antimicrobial resistances in bacteria can be achieved by phenotypic or genotypic method either qualitatively or quantitatively. In this context, the chapter will introduce you to the antimicrobial resistances, relevance in fisheries sector, methods of detecion of antimicrobial resistance, and its control measures.

Routes of transmission of Antimicrobial resistance

AMR is not a single sector problem. AMR is complex in nature and spreads across sectors such as human, animals, food and environment (Fig.2.). Finally, all the microbial populations with or without AMR enters the environment either soil or water. The picture nicely depicts transmission routes of the AMR pathogens including transboundary or intercontinental. It includes the run-off from the agriculture as well as animal agriculture.

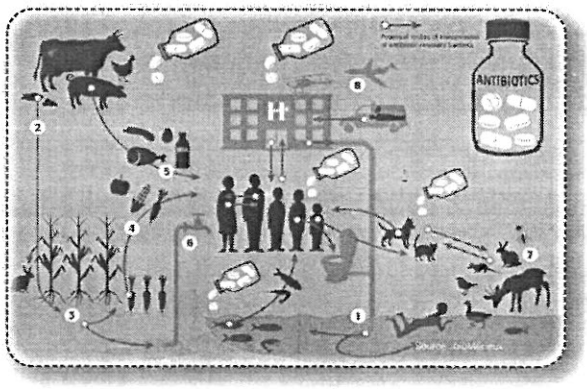

Fig. 2. Biomerieux's recent publication on probable routes of transmission of AMR pathogens across the sectors.

WHO has listed the pathogens of AMR in several categories as prioritized list of pathogens. The pathogens which are economically very important in hospital infections, community as well as food producing animals is depicted in Fig.3. The pathogens which are very relevant to fisheries sector are *Vibrios* sp, *Aeromonas* sp, and *Edwardsiella* sp.

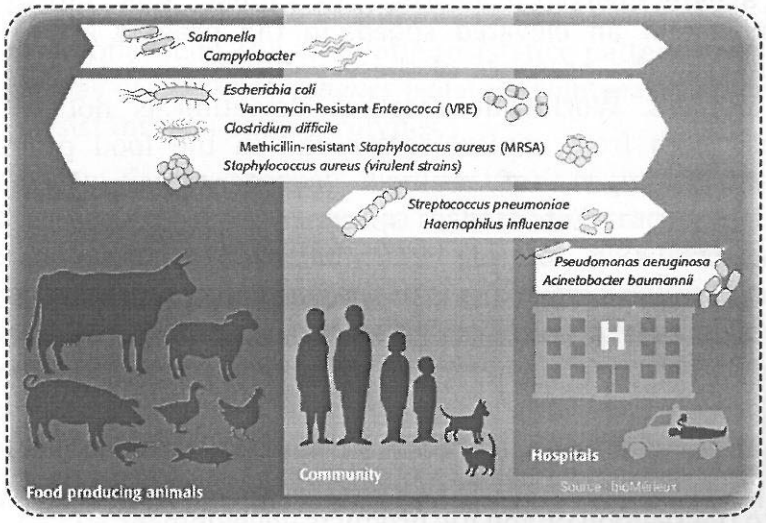

Fig.3. Pathogens of economic relevance in each sectors.

Trends in Fisheries sector

Fisheries sector plays an important role in the food security and fish is now traded internationally. Shift in the trading policies (import and export) of seafood/aquatic products are happening at a rapid pace and consumption of seafood increased globally in substantial quantum. It is estimated that aquatic products export from Asian countries outcompetes earlier contributions to their importing partner's year after year. Aquatic products include chordates, molluscs and arthropods from freshwater, brackish and marine system. They are nutrient rich diet and perishable too in nature and this prompted the industry to process the seafood in to different forms such as frozen, canned, cured and dried to extend its shelf life and recently value addition step being followed to improve the customer satisfaction. Nevertheless, the risk associated with the transboundary exchange of pathogens of seafood importance and its antibiotic resistance are generally cannot be disregarded. Majority of the pathogens are not a native flora of fish. Each step in the aquatic products production chain either in the captured or cultured fisheries involves the contact of the seafood to the environment where they are grown, various implements used, contact surfaces, handlers, water etc. This post harvest handling makes the seafood contaminated with the pathogens of seafood importance's such as *Escherichia coli*, *Salmonella* spp, *Clostridium botulinum*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahemolyticus*, *Shigella* sp, *Aeromonas hydrophila*, *Plesiomonas shigelloides* and viral pathogens such as hepatitis A virus etc. Among these pathogens, *Escherichia coli*, *Salmonella* spp, *Staphylococcus aureus* and *Shigella* spp are non-indigenous to the aquatic environment and others are indigenous to the aquatic environment. Depending on the nature of the environment (contaminated water source), feeding habits (filter feeders), season of harvest (summer season) are very crucial factors which cause seafood inherently contaminated in nature.

AMR is an increasing global public threat in various facets of healthcare system because of their rapid emergence of newer resistances and spread across the various countries. Its impact is felt across the globe. This results in prolonged illness, complications in surgical conditions due to infection with resistant organisms, severe fatal forms are also encountered. Antibiotic resistance development is a natural process occuring during due to change in genetic makeup of microbes in a longer time, however the current situation is happening at an elevated speed. In the present scenario, the risk is potentiated not only by the presence of these pathogens but also on the antibiotic resistances they possess. Worldwide research deviation is noticed on antibiotic resistant pathogens both from clinical sector and in the food producing animals. Antibiotic resistant pathogens of seafood importance are Methicillin-resistant *Staphylococcus aureus,* Extended spectrum Beta-lactamase producing Enterobacteriaceae *viz.,* ESBL *E. coli,* ESBL Salmonella; carbapenamase resistant Enterobacteriaceae viz., Klebsiella, *E. coli*; Vancomycin resistant Enterococci and so on. The link between the use of antimicrobial substances in food production and the presence of antibiotic resistant foodborne pathogens *Salmonella,* pathogenic *E. coli, Campylobacter, Staphylococcus* spp., *Enterococcus* spp. and extended-spectrum beta-lactamase (ESBL) has been already proved by various researchers. This perhaps shows the importances of studies on AMR pathogens in the food producing animals with special reference to the seafood or aquatic products development.

In general to exception of commercially sterile and other pro,pre and synbiotics food products, food have the proximity of getting contaminated to various microbes during entire production and processing chain. The raw food in general have the highest culturable bacterial concentrations, followed by minimally and fully processed foods. Minimally or fully processed food including ready-to-eat food contamination depends on the level of sanitary hygiene followed during the processing and preservation steps (Fig.4.).

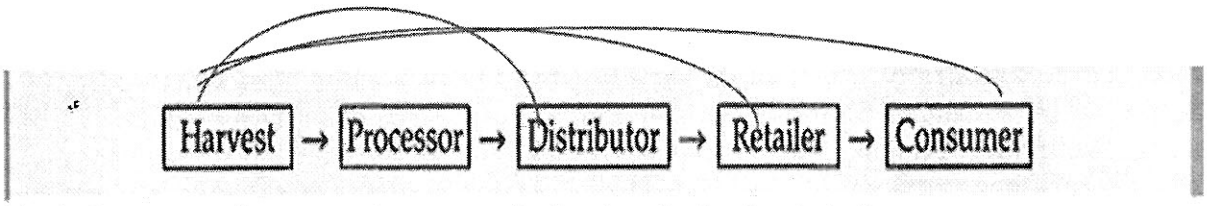

Fig.4. Steps contributes to the entry of microbes in the food chain

The food with acceptable microbiological quality range may also serve as the sink for the development of antibiotic resistances through bacteria, bacteriophages, bacterial DNA and mobile genetic elements, some of which may include AMR genes. Hence, the food chain ecosystem may be conducive niches for gene transfer, selection and persistence of AMR bacteria and this route cannot be generally disregarded.

V. parahaemolyticus, V. vulnificus, V. alginolyticus, and V. cholerae are autochthonous Gram-negative bacilli to estuarine and marine environments and found associated with disease through wound infection or through consumption of contaminated seafood especially shellfish. Antimicrobial resistant Pathogenic bacteria released into aquatic environments through wastewater acts as potential spread of antibiotic resistant genes spread. In general Vibrios sp showed higher resistances towards Ampicillin and low

tetracycline resistances. The frequency of resistance reported in aquatic products ranged from 16.6 to 50% level and 10 to 69% of the vibrio strains showing resistance to more than 4 molecules. Common antibiotics showed resistances are teicoplanin, pencillin, oxacillin, vancomycin and low level resistance for cephalosporin groups.

Highly resistant to penicillin, ampicillin, tetracycline, and vancomycin was observed in *L. monocytogenes* isolated from seafood and low level less than 10% for Tetracycline, enrofloxacin, and ciprofloxacin. The antibiotic resistance pattern and number changes between the serotypes of *L. monocytogenes* isolated from seafood, serotype 1/2a was found to be more resistant than other serotypes.

S. aureus isolated from fishery products were resistant to penicillin, chloramphenicol and ciprofloxacin and most of them were also resistant to tetracycline. In general, to the β-lactams, Macrolides, aminoglycosides, ciprofloxacin, co-trimoxazole (4.7%) and tetracycline resistances were observed in most of the studies with varied percentage. Pencillin, Macrolides are above 50% and others were less than 50% level. Multidrug resistant strains were also reported in many studies.

Salmonella isolated from seafood were in general resistant to the pencillin, erythromycin, tetracycline and other antibiotics were less than 15% level. In a study conducted on imported seafood in to US from 20 countries, S. enterica strains of 36 serovar were isolated and twenty isolates showed resistance to at least one antibiotics. Five strains (serovars Bareily, Oslo, Hadar, Weltevreden and Rissen) were resistant to two or more antibiotics. Two *S. enterica* strains (serovars Bareily and Oslo) from seafood from Vietnam and India were resistant to trimethoprim/sulfamethoxazole, sulfisoxazole, ampicillin, tetracycline and chloramphenicol. Multidrug resistant strains were also observed in Salmonella isolated from seafood.

In addition to this, Fish are reservoirs for zoonotic pathogens not only infecting the host animal but also humans in contact during aquaculture activity.The infections includes *Aeromonas hydrophilia, Mycobacterium marinum, Streptococcus iniae, Vibrio vulnificus,* and *Photobacterium damselae* etc are noted few.

All the study demonstrated that there is a change in the trend of antibiotic resistances which depends on the country of origin of the seafood, antibiotic usage in particular country for aquaculture practices etc.

Work done at ICAR-CIFT

ICAR-CIFT has been working in four states of India viz., Kerala, Andhra Pradesh, Maharastra and Gujarat for the past five years on AMR. *Staphylococcus aureus, Escherichia coli, Vibrio cholerae, Vibrio parahaemolyticus, Listeria monocytogenes and others.* Some of the important findings in AMR in public health significance bacteria are

The prevalence of MRSA (Methicillin resistant *Staphylococcus aureus*) was 13.8%, 9.3%, 12%, 15.3% in fish, crustaceans, molluscs and environment samples collected from Kerala, respectively. *spa* (Staphylococcal protein A) typing of the MRSA isolates revealed that MRSA from the fish landing centre (t311 and t15669) was carried to the retail fish market. T15669 Novel clone identified in the landing centre. Coagulase positive Staphylococci isolated from seafood samples from Veraval, Gujarat demonstrated resistance to at least three groups of antibiotics (multidrug resistance – MDR). 97.67% of the *S. aureus* isolates were resistant to azithromycin, ciprofloxacin and gatiflaxacin while 93.33% were resistant to lomefloxacin. Antimicrobial resistance to

Ceftriaxone, a third-generation cephalosporin antibiotic, was found in *L. monocytogenes* isolate from fish.

The antibiotic resistance profile of 382 *V. cholerae* isolates from seafood samples from Kerala revealed that 26, 40, 62 and 84% of non 01 and non 0139 strains were resistant to Cefpodoxime, Ticarcillin, Augmentin and Colistin, respectively. *V. cholerae* isolates from Andhra Pradesh revealed that 37.5% isolates were resistant to cefotaxime, and 12.5% of the isolates were resistant to ceftriaxone and nitrofurantoin. However, all the *V. cholerae* isolates were sensitive to 21 other antibiotics. Multidrug resistant *Aeromonas hydrophila* was recovered from fish farms.

Methods of detection of Antimicrobial resistance

In general, the methods involved in the determination of antimicrobial resistances are categorized into phenotypic and genotypic and phenotypic further classified into qualitative as well as quantitative (Fig. 5)

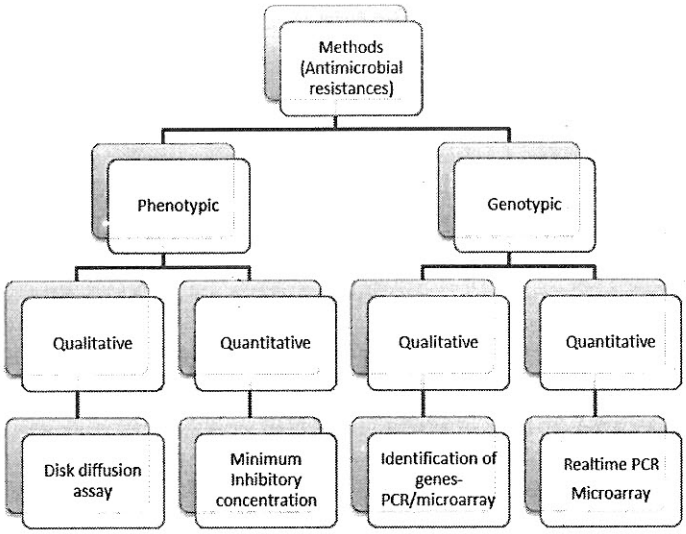

Fig.5. Methods of determination of antimicrobial resistances

Qualitative methods

Phenotypic methods

Disk diffusion assay

Bauer, Kirby, Sherris, and Turck harmonized the procedure of disk diffusion by controlling the variables such as the media, temperature, and depth of agar and published the detailed paper in 1966.

Detailed steps involved in the disk diffusion assay

Selecting the colonies: Preparing the inoculum involves pick or selecting 3–5 well-isolated colonies using an inoculating loop or a cotton swab from the primary plate, suspending them in broth or standardizing the suspension. Avoid testing mixed cultures or subculture the organism to a fresh plate for purity checking.

Prepare inoculum suspension: The turbidity of the test suspension from Direct colony (not more than 18–24 hours) or log phase growth (Broth culture approximately 8

hours) in saline or Mueller hinton or Tryptic soy broth standardized to match that of a 0.5 McFarland standard (approximately 1.5 X 10^8 CFU/ml) and use inocula within 15 minutes. Compare the turbidity of the suspensions by placing the tubes in front of a white paper or file card with black line. Direct colony suspension is commonly recommended for Staphylococci and Streptococci. Log phase culture for most of the non-fastidious microorganism. In general avoid over grown cultures or old cultures.

In general Mueller Hinton agar is prepared as pre-set with depth of 4 mm at least, dried, kept at incubator for 10-15 min to absorb excess moisture in the plate. Bring the antimicrobial disk to the room temperature 1-2 hour before impregnating to reduce the condensation. Vortex the suspension to mix well and dip a fresh, sterile cotton-tipped swab into the suspension. Remove the excess liquid from the swab by pressing it against the side of the tube. Inoculate the surface of the MHA plate to cover the entire plate back and forth and from edge to edge, then rotate the plate approximately 60° angle, repeat the procedure two times and finally encircle it, so as to ensure the even distribution of the inoculum.

Apply antimicrobial disk within 15 min and incubate the plate within 15 min. 12 and 5 disks can be applied to a 150 mm and 100 mm diameter plate respectively. Alternatively the disk can be applied with the dispenser. Invert and incubate plates with agar side up, for nonfastidious bacteria in general incubate in ambient air at 35°C for 16–18 hours and for fastidious organism and other environmental bacteria incubate as appropriate to the desired organism.

After incubation, examine plates for the lawn of growth is even and confluen and clear unobstructed zones. Measure zones from the back of the plate using reflected light by holding the plates a few inches above a black non-reflecting surface using vernier caliper or zone measurement ruler. Reflected light for all the organism and transmitted light for vancomycin or oxacillin resistant Staphylococci or Enterococci.

Plates with fuzzy or double zone lines or feathered edges or swarming and colonies inside zones should be taken adequate care in reporting. This occurs in mixed culture or resistant subpopulation within the culture. Measure the inner most zone for double zone. Pick the inner colony and check for the purity and repeat the antimicrobial susceptibility testing once again. In general colony free zone is taken. For feathered edge the region demarcating growth and no growth is taken as zone region. Ignore generally swarming and measure the zone region only.

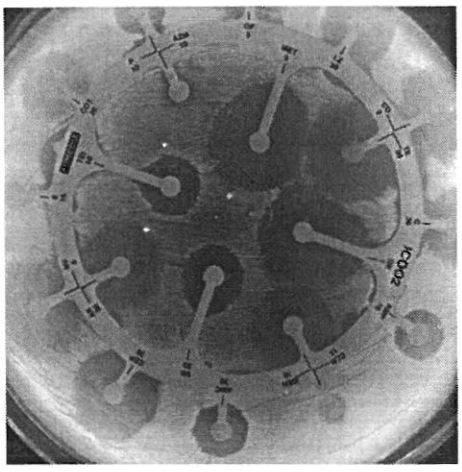

Fig.6. Results of Disk diffusion assay

Quantitative methods

MIC

The minimal inhibitory concentration (MIC) of an antimicrobial agent is the lowest (i.e. minimal) concentration of the antimicrobial agent that inhibits a given bacterial isolate from multiplying and producing visible growth in the test system. MIC tests can be performed in broth or agar media dilution or broth microdilution in microtitre plates.

Broth microdilution MIC is performed in a polystyrene panel containing approximately 96 wells which may be sufficient enough for a panel of 12 antibiotics upto 7–8 dilutions with one well each for a positive growth control (broth plus inoculum) and negative control (broth only). In general volume of 0.1 or 0.2 mL in each well the MIC is performed. Use separate panel for gram-positive bacteria and gram-negative bacteria and special media for fastidious bacteria. In general, Mueller-Hinton broth with appropriate divalent cation (Ca++ and Mg++) is recommended for susceptibility for rapidly growing aerobic, or facultative organisms non-fastidious organism. The pH of the medium adjusted to 7.2 and 7.4 at room temperature (25°C). For fastidious organisms Mueller-Hinton broth may be supplemented with 2–5% lysed horse blood or according to the organism.

In the agar dilution method, the antimicrobial agent is incorporated at different concentration into the each agar medium Mueller-Hinton agar. The pH of the agar must be between 7.2 and 7.4 at room temperature. The inoculum is applied on surfaces as replicators transfer 32–36 inocula to each plate.

Follow the same procedure of inoculum suspension preparation as mentioned in disk diffusion assay. Within 15 minutes of adjusting the inoculum to the 0.5 McFarland turbidity standard, dilute it further so that the final concentration in each well is 5×10^5 CFU/mL. Deliver 2.0 mL of the original suspension into 38 mL of water (1:20 dilution). Inoculate 0.01 mL (1:10 dilution) into each well and finally inoculate MIC panel carefully to avoid splashing from one well to another. Cover the plate to avoid dehydration during incubation. Incubate non-fastidious bacteria in ambient air at 35°C from 16–20 hours. Staphylococci incubate oxacillin and vancomycin for 24 hours. For vancomycin, high level gentamicin resistance (synergy test) and high level streptomycin resistance (synergy test) for 24 hours.

At the end of the testing, check the plate for purity. Check the positive control well for growth of turbidity or a button of growth >2 mm should be present. Check the negative control well for no growth or clear. Read the MIC endpoint as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye. Always include the MIC breakpoint for the particular organism for that particular antibiotic.

The performance of each batch of broth or antimicrobial are evaluated with standard set of quality control organisms.

Other commercial manual and automated systems used for determination and quantitation of phenotypic antimicrobial resistances are E-test,l vitek system, microscan etc

Genotypic methods

Looking for genes involved in the conferring resistance to antibiotics are now currently more followed in well established laboratories. The detection of mechanism of

antibiotic resistance can be better elucidated from the genes present in the organism. This is achieved by polymerase chain reaction targetting against genes involved in each class or generic or group of antibiotics. Over 30 class and subclass of antibiotics are available. Resistance mechanism developed against these antibiotics were mediated by over 100 different genes and their mutants or variants. In general, the PCR is targeted against these genes to look for the possible molecular mechanism involved in the acquiring of antibiotic resistances. Even the researchers moved to the quantitation of antibiotic resistance for particular antibiotics by a pathogen using realtime PCR or microarray analysis.

Control

AMR is a complex and indisciplinary issue, coherent efforts are required to bring down the burden of AMR among public. WHO, FAO and OIE have taken collective tripartite one health approach to control AMR spread which are considered as national action plans to each countries. The proposed the action plan against AMR control is depticted in Fig.7 (WHO).

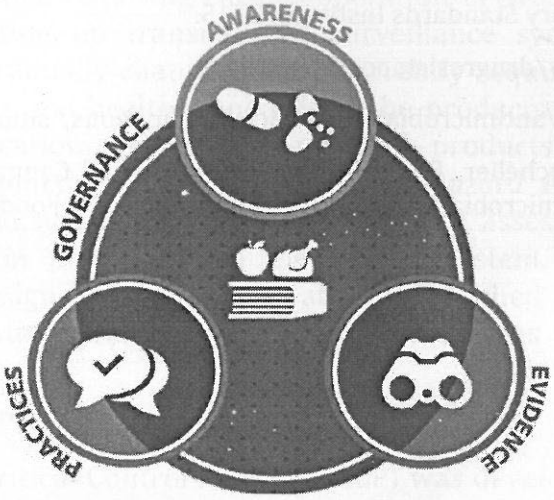

Fig.7. The four pillars of the FAO plan of action to support the food and agriculture sector in addressing AMR

Key action plans proposed to control AMR are

1. Strengthen the surveillance system in healthcare, food producing animals on antimicrobial usage and antimicrobial drug resistant bugs
2. Emphasis need to be given to the food and environmental sectors also
3. Strengthening the laboratory capacity for surveillance system
4. Guideline for the optimised use of antibiotics in human and animal health
5. Reduce the infection loss due to AMR pathogens by providing assured quality medicines
6. Awareness and understanding among the general public
7. Effective infection prevention and control programmes
8. Development of alternate to antibiotics protocols

9. Controlling the resistances development in bacteria for medically important antibiotics

Whole world is looking for alternatives to the antibiotic in combating the bacterial infections which may consequently reduce the amount of antibiotic used in therapies and lower doses in other animal agriculture practices. Many alternatives are looked in and few promising among them are lytic phages, probiotics, antimicrobial peptides, quorum quenching molecules etc.

Suggested reference

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