GENOME-WIDE ASSOCIATION STUDY: A GENOMIC RESORT TO TACKLE THE MENACE OF ANTIMICROBIAL RESISTANCE

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Antimicrobial resistance (AMR) has been identified as a serious calamity having widespread ramifications for animal, human as well as environmental health. For tackling AMR, along with antibiotic stewardship and measures for controlling and preventing diseases, there is a need to look into the genetic aspect of diseases. Disease resistance is a highly complex trait with various underlying causative variants, genes and biological pathways responsible for it. So, a deeper look into the genomic information could offer insights for selecting animals for disease resistance. A Genome-wide Association Study (GWAS) is well suited for this purpose as it brings to the fore, genomic variants and regions associated with disease resistance by exploiting variability among the individuals. Once the actual causative mutations are identified for important animal diseases by GWAS, selection and consequently, raising of animals for resistance against a disease will become a lot easier. This in turn, will contribute to lesser reliance on antimicrobials and hence, could serve as an important and effective weapon to root out the phenomenon of antimicrobial resistance.

Key words: Antimicrobial resistance, Disease resistance, GWAS

Animal health is an indispensable component encompassing production, reproduction and animal welfare aspects (<http://www.fao.org/animal-health/en/>; Wolfenson *et al.*, 2015; <https://awionline.org/sites/default/files/uploads/documents/FA-AWI-Animal-Health-Welfare-Report-04022018.pdf>). Anti-microbial drugs have served as an important pillar of animal health support systems by playing an important role in therapeutics, metaphylactics, prophylactics and growth promotion (Woolhouse *et al.*,

2015). Poor quality antibiotics, ill-trained veterinary practitioners and rising demand for meat are some of the factors which have further pushed the reliabilities on antimicrobial drugs (Clifford *et al.*, 2018; Wegener, 2012; Walia *et al.*, 2019). All this has led to the emergence of the phenomenon of antimicrobial resistance (AMR) which has been dubbed as a worldwide calamity (Kunin, 1993) with far reaching implications on animal, human as well as environmental health (Taneja and Sharma, 2019). In fact,

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combating the menace of antimicrobial resistance was an important reason behind the genesis of 'One Health' concept which aims at harmonisation of strategies and policies directed at humans, animals as well as environment and adopting a coordinated as well as integrative approach for the improvement of health of all (<https://www.oie.int/en/for-the-media/editorials/detail/article/one-health/>; Woolhouse *et al.*, 2015; Marquardt and Li, 2018).

The grim scenario regarding indiscriminate use of antibiotics is depicted by the fact that more than 70% of all the antimicrobial drugs sold in the world are used on food animals (Boeckel *et al.*, 2019). This further led to the proposition that livestock are acting as repositories of resistance genes (Woolhouse *et al.*, 2015) and these genes are entering humans through the food chain, thus prolonging the illness, frequent bouts of illness, emergence of highly resistant strains of microorganisms (Kumarasamy *et al.*, 2010) and consequently, failure of treatment (https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/amrfoodchain/en/). However, a recent study has demonstrated that the gene transfer is in fact, happening in the other direction as well and the resistant genes are also getting introgressed from humans to other species (Richards *et al.*, 2019).

India and China have been reported as the global hotbeds for AMR in livestock owing to the large livestock population and burgeoning demand for livestock products (Boeckel *et al.*, 2019; https://www.reactgroup.org/wp-content/uploads/2018/11/Antimicrobial_Resistance_in_Food_Animal_Production_India_

[Overview_2018_web.pdf](#); Laxminarayan and Choudhary, 2016). A study collating 901 point-prevalence surveys revealed startling figures: the proportion of antimicrobial drugs with resistance higher than 50% (P50) in low and medium income countries like India and China, increased dramatically in case of chickens (173%) and pigs (161%) between 2000 and 2018 (Boeckel *et al.*, 2019). It has made the treatment of sick animals and production of enough meat to satisfy the demands of population, increasingly difficult (Bengtsson and Greko, 2014). The reported estimates peg the direct costs of individual diseases to be 35-50% of the total turnover in the developing countries (Bennett, 2003).

Selection for disease resistance in livestock

Besides environmental influences, the resistance of an animal to diseases is also dictated by the genetic makeup of the individual. Long term and sustainable resistance in a population against a particular disease is often achieved by exploiting existing genetic variability among the individuals for resistance against that disease which would also complement other environmental interventions (Gibson and Bishop, 2005; Bishop, 2010). Selection for disease resistance is not a new concept and the awareness regarding this, dawned on the animal breeders long back. Since then, the research was targeted at selection for improving various livestock species against particular diseases - nematodiasis in sheep (Stear *et al.*, 1997; Bisset and Morris, 1996), mastitis in cattle (Heringstad *et al.*, 2000), enhanced immune responsiveness in pigs (Mallard *et al.*, 1998; Wilkie and Mallard, 2000) and avian lymphoid leucosis complex

and Marek's disease in chickens (Cole, 1968). In this respect, various studies have also emphasised on the lower susceptibility of indigenous Zebu cattle against diseases and parasitic infestations, as compared to exotic cattle breeds (Vordermeier *et al.*, 2012; <https://pdfs.semanticscholar.org/1dfe/8cc8349dfe0c5e3040167a2f84346463211e.pdf>) and this has been attributed to the variability in the genetic makeup (<http://www-naweb.iaea.org/nafa/aph/crp/aph-improving-animal-productivity.html>). The traditional approaches for breeding of livestock for disease resistance included: selecting sires and/or animals successively in each generation (for lesser disease incidences) for future breeding (Heringstad *et al.*, 2007; Tsairidou *et al.*, 2019) and identification of MHC antigens involved in immune responses and selecting individuals with a particular antigen indicative of resistance to a disease (Bearse *et al.*, 1963; Lamont, 1998). With the advent of advanced molecular techniques, expression profiling was carried out to explore the candidate genes responsible for disease resistance (Abdul-Careem *et al.*, 2007; Taylor *et al.*, 2008; Fonseca *et al.*, 2009) and further association studies were undertaken in a quest to identify DNA markers for selection by targeting the association between candidate genes polymorphisms and indicators of resistance and/or susceptibility for specific diseases (Ibeagha-Awemu *et al.*, 2008; Wu *et al.*, 2008; Ogorevc *et al.*, 2009; Verschoor *et al.*, 2010).

Genomics wave in animal breeding

Since early 2000s, a new 'genomics era' has dawned in animal breeding with the

emergence of high-throughput genotyping platforms and next-generation sequencing technologies and the costs of genotyping are also falling considerably (Eggen, 2012). As a result of this, selection of animals has also witnessed an evolution from phenotypic selection to marker-assisted selection and consequently, to genomic selection. The underlying principle in genomic selection is that if markers throughout the genome are targeted for selection, there is higher chance of accounting for most of the genetic variance for a trait as the quantitative trait loci (QTL) or underlying causative mutation responsible for the trait would be in linkage disequilibrium (LD) with at least one marker (Meuwissen *et al.*, 2001; Goddard and Hayes, 2007). Also, there is a growing realisation that breeding animals for major disease resistance genes is effective but these genes are highly vulnerable to breakdown with rapid changes in the pathogenic races and that the breeding objective should shift towards understanding the genetic architecture of many diffused minor genes. This necessitates genomic-assisted breeding for quantitative resistance using genomic prediction models and selection (Poland and Rutkoski, 2016). Unravelling the genetic architecture of a disease by identification and validation of genomic regions conferring disease resistance would facilitate the propagation of resistant animals through genomic selection (Yuan *et al.*, 2018). In this context, a Genome-wide Association Study (GWAS) is a very useful tool to improve the accuracy of genomic selection by shedding light on the identified genetic markers, putative genes and QTL regions underlying the complex mechanisms of disease resistance

and hence, act as an initial screening tool (Meredith *et al.*, 2012; Zhang *et al.*, 2014a; Lee *et al.*, 2015b).

Genome-wide Association Study

Till date, more than 10,000 loci have been identified for common heritable human diseases and traits using a highly powerful approach – Genome-wide Association Study (GWAS), which is a hypothesis-free tool to reveal the associations between genomic regions (SNPs, genes, QTLs) and traits (diseases and quantitative traits) (Duncan and Brown, 2018). The GWAS have also been widely employed in various livestock populations to explore the genetic variants related to economic traits viz. production, functional and conformation traits (Cole *et al.*, 2011; Wang *et al.*, 2018; Gerlando *et al.*, 2019). The principle behind a Genome-wide Association Study is to detect the markers throughout the genome (usually SNPs) having a significant p-value by testing their association with the trait of interest (Zhang *et al.*, 2014a; Schmid and Bennewitz, 2017).

Identification of Quantitative Trait Nucleotide (QTN) or underlying causative mutation is the end product desired from genomics so as to make the selection based on genomic information more precise as well as cost-effective (Weller and Ron, 2011). GWAS makes it a lot easier by casting light on the potential regions where to look up for the causative variants by fine-mapping (Weller, 2016). Also, the accuracy of genomic breeding values (GEBV) obtained based on GWAS results are higher than those obtained solely on the basis of whole genome prediction (Zhang *et al.*, 2014b).

Genomic data obtained using various SNP arrays usually serves as the raw material for GWAS although the accuracy of results depends on the marker density used, effective population size, heritability and genetic architecture of the trait (Matukumalli *et al.*, 2009; Liu *et al.*, 2017; Zhang *et al.*, 2019). In a GWAS, trait associations with SNP markers could be estimated either with phenotype of the trait (Bouwman *et al.*, 2011; Yodklaew *et al.*, 2017) or with its pseudo-phenotype, which is a projection closest to the genotypic estimate (Liu *et al.*, 2004; Garrick *et al.*, 2009).

Quality control in GWAS

A basic requisite before determining actual association between genomic and phenotypic information in a GWAS is the quality control (QC) of the genomic data (Turner *et al.*, 2011). For QC, the trimming of genomic data is done based on certain criteria usually, call rate (CR), minor allele frequency (MAF) and test of Hardy-Weinberg equilibrium (HWE) (Liu *et al.*, 2018). Call rate is an important criterion for QC of both SNPs and samples (Jiang *et al.*, 2010). For a given SNP, CR is defined as the percentage of individuals for which the information of that SNP (called SNP) is not missing and sample call rate excludes individuals with higher percentage of missing genotype data out of the total typed SNPs (Reed *et al.*, 2015). Most of the studies set the pre-defined threshold of $\geq 90\%$ for SNP as well as sample filtering (Meredith *et al.*, 2012; Wu *et al.*, 2014; Abdoli *et al.*, 2018). Another important criterion is minor allele frequency. The MAF is defined as the frequency of less common allele at a locus in the population and most of the studies

employ ≥ 0.05 as cut-off value for SNP to be included for further analysis (Fan *et al.*, 2011; Nazari-Ghadikolaei *et al.*, 2018). Violation of Hardy-Weinberg equilibrium is expected due to differing population structure and genotyping error (Reed *et al.*, 2015). So, the SNPs showing departure from HWE (usually p value $\geq 10^{-6}$) are not included in the final analysis (Jiang *et al.*, 2010; Lee *et al.*, 2015b).

Statistical analysis in GWAS

For testing association between SNP markers and the dependent trait, two types of association models are used: Single-marker models and Multi-marker models

Single marker models: Here, one SNP is fitted at a time in the model with the null hypothesis of no association between SNP and the trait (Schmid and Bennewitz, 2017). In the context of GWAS, single-marker model approach is more commonly utilised (Balding, 2006). However, due to multiple tests being performed simultaneously, there is a serious inflation of type-I error, which can lead to thousands of false-positive associations even when none is actually associated with the trait (Zeng *et al.*, 2015). Due to this, another problem of ‘Beavis effect’ crops up, which is the over estimation of the true QTL effects of statistically significant associations (Beavis, 1994; Weller, 2016).

A possible way out to handle the problem of multiple comparisons in GWAS include Bonferroni correction and false discovery rate (FDR). Bonferroni correction gives a conservative estimate by setting strict significant thresholds to find false positives. Here, the adjusted p-value is estimated as:

α/m where, α = significance level set (typically 0.05) and m = number of markers used. However, it is not able to account for most of the genetic variance for a trait as most of the SNPs fail to meet the significance criterion (Zeng *et al.*, 2015). On the other hand, false discovery rate assesses the number of associations reaching significance thresholds, which are actually false positives (Benjamini and Hochberg, 1995). FDR gives a q-value threshold as the proportion of significant features which turn out to be false positives (Storey and Tibshirani, 2003). It is calculated as $FDR = m \alpha / i$ where, m = number of markers (SNPs) tested for a trait, α = p-value or level of significance utilized, i = no. of cases in which the null hypothesis got rejected at that p-value (Schmid and Bennewitz, 2017).

Multi-marker models: In another attempt to address the problem of multiple testing and number of SNPs disproportionately exceeding the sample size, multi-marker models were devised which though originally developed for genomic selection (Meuwissen *et al.*, 2001), became highly popular in GWAS studies as well (Sahana *et al.*, 2011; Goddard *et al.*, 2016). The basic principle of multi-marker models is that all SNPs are fitted simultaneously as random effects. Based on the assumption regarding the distribution of marker effects, different types of multi-marker models are used (Schmid and Bennewitz, 2017).

The SNP-BLUP models and GBLUP models invoked the infinitesimal model of genetic variation to estimate marker effects, assuming a normal distribution with a small variance for each marker. The former

employs SNP-SNP relationship matrixes (Lee *et al.*, 2015b) while the latter employs genomic relationship matrix (GRM) to generalise marker effects even for individuals without genotypic data (Aguilar *et al.*, 2010; Wang *et al.*, 2012).

Conversely, several Bayesian models have been proposed which assume unequal variances across SNPs and employ models with differing prior distribution of SNP variances. Bayes A model assumed a thicker t-tailed distribution of marker effects while Bayes B considered a t-distribution for only a fraction of SNPs which were assumed to contribute towards the variance of a trait (Meuwissen *et al.*, 2001). Bayes C considers two SNP fractions- one with a larger variance and another with a smaller variance for the trait and hence, assuming two different t-distributions for each (Verbyla *et al.*, 2009; Verbyla *et al.*, 2010). Bayes R assumes a mixture of normal distribution for certain effective SNPs and allows few SNPs with large effects (Erbe *et al.*, 2012). Bayesian LASSO assumes different variances for residual and SNP effects (Legarra *et al.*, 2011).

The SNPs which are found to be statistically significant in GWAS are represented in a Manhattan plot which is a scatter plot having genomic coordinates in X-axis and negative logarithm of p-value for association (between SNP and trait) on Y-axis (https://en.wikipedia.org/wiki/Manhattan_plot). Another graphical representation of GWAS results (particularly, association models for diseases) is a QQ plot in which the observed and the expected p-values are plotted from a theoretical Chi-square distribution (Ehret, 2010).

GWA studies for disease resistance in various livestock species

A successful GWAS was first reported in 2002 in a human study for myocardial infarction (Ozaki, 2002). This was soon followed up by a landmark study in 2005 for screening genetic variants related to age-related macular degeneration in humans (Klein *et al.*, 2005). While in humans, the GWAS were directed mostly on disease susceptibility and studies on height (Hill, 2012), in animals, these studies took a more diversified approach to study economic quantitative traits in addition to disease resistance (Cole *et al.*, 2011; Sahana *et al.*, 2014). Selecting animals which are resistant against particular diseases improves the overall production, reproduction, health and welfare prospects (Rexroad *et al.*, 2019).

In cattle, mastitis is one of the most commonly studied diseases based on GWAS data due to its multi-factorial nature and huge economic implications (Meredith *et al.*, 2013; Sahana *et al.*, 2014; Wang *et al.*, 2015). Somatic cell count (Kurz *et al.*, 2018; Welderufael *et al.*, 2018) and somatic cell score (Meredith *et al.*, 2012; Wang *et al.*, 2015) are commonly used as indicators of mastitis susceptibility in most of the studies. Similarly, studies have been conducted to identify important markers and loci associated with infectious diseases like tuberculosis and foot and mouth disease (Bermingham *et al.*, 2014; Alpay *et al.*, 2014; Lee *et al.*, 2015a). In small ruminants like sheep and goat, nematode resistance is often the targeted trait through GWAS (Wilkie *et al.*, 2017; Zvinorova, 2017). Pigs, being mostly afflicted with respiratory afflictions, have been evaluated genome-

Table 1. GWAS carried out in different livestock species for disease resistance

Species	Breed	Diseases or traits considered	Number of significant SNPs and /or candidate genes/ QTL region reported	References
Cattle	Holstein	Foot and mouth disease	11 SNPs MYO18B and SEZ6L genes	Lee <i>et al.</i> , 2015a
	Holstein-Friesian	Bovine tuberculosis	3 SNPs SLC6A6 gene	Finlay <i>et al.</i> , 2012
	Holstein	Johne's disease	SLCO6A1, ILI2B, TIMD4, C5, CD44 genes	Alpay <i>et al.</i> , 2014
	Holstein	Mastitis	117 SNPs and 27 QTLs	Kurz <i>et al.</i> , 2018
	Holstein-Friesian	Tuberculosis	PTPRT and MYO3B genes	Birmingham <i>et al.</i> , 2014
	Chinese Holstein	Mastitis	48 SNPs TRAPPC9 and ARHGAP39 genes	Wang <i>et al.</i> , 2015
	Canadian Holsteins	Immune response traits	BoLA-DQ, BoLA-DR, BoLA-NC1, BoLA-DQ, galectins 1, 2 and 3, BCL2 and α -defensin genes	Thompson-Crispi <i>et al.</i> , 2014
	U.S. Jersey	Ketosis	TTLL7, ARAP2, CD207, SPAST, FERMT2, BMP4, DDHD1, XDH genes	Gaddis <i>et al.</i> , 2018
	Canadian Holstein	Subclinical ketosis	SLC4A4, FRAS1, GC, FRAS1, CXCL8, RASSF6, FAM47E genes	Nayeri <i>et al.</i> , 2019
Sheep	Gir X Holstein crossbreds	Tick infestation	TREM1, TREM2, CD83, MYO5A, TREML1 and PRSS16 genes	Otto <i>et al.</i> , 2018
	Scottish Blackface	Nematode resistance	12 SNPs IL23R, RORC2 and TBX21 genes	Wilkie <i>et al.</i> , 2017
	Corriedale	Inherited rickets	DMP1	Zhao <i>et al.</i> , 2011

Cont. Table 1.

Species	Breed	Diseases or traits considered	Number of significant SNPs and /or candidate genes/ QTL region reported	References
	Scottish Blackface	Nematode resistance	3 SNPs	Riggio <i>et al.</i> , 2013
	Texel	Foot rot	7 SNPs	Mucha <i>et al.</i> , 2015
Goat	Breed not specified	Gastrointestinal parasites	15 SNPs ORC5, RELN and NALCN genes	Zvinorova, 2017
	Creole	Gastrointestinal nematodes	13 QTLs	La Chevrotiere <i>et al.</i> , 2012
Pig	Chinese Erhualian pigs	Swine respiratory disease	CXCL6, CXCL8, KIT and CTBP2 genes	Huang <i>et al.</i> , 2016
	Landrace	Respiratory disease immune capacity	5 QTLs 18 QTLs	Okamura <i>et al.</i> , 2012
	Large White pigs	Metabolism and protein related traits leukocyte traits	13 SNPs 11 SNPs	Bovo <i>et al.</i> , 2019
Chicken		Salmonella pullorum infection	43 SNPs	Xiao <i>et al.</i> , 2019
		New Castle disease virus	2 SNPs ROBO2 gene	Luo <i>et al.</i> , 2013
		Marek's disease	Regions on chromosomes 2,3,4 9,15,18 and 21	Wolc <i>et al.</i> , 2013
		Heterophil/ lymphocyte ratio traits	15 SNPs CARD11, BRIX1 and BANP	Zhu <i>et al.</i> , 2019

wide for important markers and genomic regions related to it (Huang *et al.*, 2016) while in poultry, markers and QTL regions have been identified for various infectious diseases using GWAS (Luo *et al.*, 2013; Wolc *et al.*, 2013; Xiao *et al.*, 2019).

These days, researchers are going for meta-

analysis combining the information from earlier available GWA studies to draw more concrete and substantial conclusions (Minozzi *et al.*, 2012; Blaj *et al.*, 2018). A review of GWAS for disease resistance in various livestock species has been presented in Table 1.

GWAS and the issue of antimicrobial resistance

Tackling the menace of antimicrobial resistance requires a multi-pronged strategy which should not only be targeted at antibiotic stewardship and infection control measures (Toner *et al.*, 2015) but also at exploring the variability at the genomic level for resistance against various diseases (Caballero *et al.*, 2018; Farhat *et al.*, 2019). However, genetics of disease resistance is a complex trait determined by multiple factors and interactions (<https://www.nature.com/scitable/topicpage/complex-diseases-research-and-applications-748/>), which are not fully accounted by linkage studies (https://en.wikipedia.org/wiki/Genome-wide_association_study). A GWAS is a more powerful way to trace out the genomic variants and locations where the key to resistance against a disease may be hidden (Hirschhorn and Daly, 2005). Several GWAS directed at various diseases in livestock have already been undertaken and useful information has been obtained (Sharma *et al.*, 2015). Selection of animals

against various diseases based on GWAS results could be an important component of fight against antimicrobial resistance and would surely play its part in spelling doom for it (Chen *et al.*, 2015).

Conclusion

With the farming becoming more intensive and profit-oriented, the incidences and frequency of diseases are going to rise. This, accompanied by indiscriminate antimicrobial use and consequent emergence of highly resistant organisms is going to make the matters worse. In this scenario, relying solely on prophylactic and therapeutic measures (or any other environmental manipulation) is not enough and long-term sustainable resistance against diseases is possible only through the improved genetics of animals. In this context, genome-wide association studies could be of great help in pinpointing the underlying genetic causes of disease resistance and/or susceptibility and hence, could pave the way for selection of resistant animals in future.

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