**Spotlight on avian pathology: *Eimeria* and the disease coccidiosis**

Damer P. Blakea,\*, Virginia Marugan-Hernandeza, Fiona M. Tomleya

aPathobiology and Population Sciences, Royal Veterinary College, Hawkshead Lane, North Mymms, AL9 7TA, UK

dblake@rvc.ac.uk; vhernandez@rvc.ac.uk; ftomley@rvc.ac.uk

\*Corresponding author

**Abstract**

Coccidiosis caused by *Eimeria* species parasites remains a major threat to poultry production, undermining economic performance and compromising welfare. The recent characterisation of three new *Eimeria* species that infect chickens has highlighted that many gaps remain in our knowledge of the biology and epidemiology of these parasites. Concerns about the use of anticoccidial drugs, widespread parasite drug resistance, the need for vaccines that can be used across broiler as well as layer and breeder sectors, and consumer preferences for ‘clean’ farming, all point to the need for novel control strategies. New research tools including vaccine delivery vectors, high throughput sequencing, parasite transgenesis and sensitive molecular assays that can accurately assess parasite development *in vitro* and *in vivo* are all proving helpful in the ongoing quest for improved cost-effective, scalable strategies for future control of coccidiosis.

Keywords: Eimeria; Chickens; Economic impact; New species

***Eimeria*, cause of the disease coccidiosis**

*Eimeria* are protozoan parasites that can cause coccidiosis in all livestock and poultry, in addition to many other non-production species. Most *Eimeria* species are strictly limited to a single host-species, although examples such as *Eimeria innocua* from domestic turkeys can also replicate in grey partridge and bobwhite quail (Vrba & Pakandl, 2015). Seven *Eimeria* species have long been recognised to infect chickens, appearing on every continent where chickens are farmed (Clark *et al.*, 2016). Additionally, three cryptic *Eimeria* genotypes have recently been identified as new species (Blake *et al.*, 2021; Cantacessi *et al.*, 2008). At least five *Eimeria* species infect turkeys, although their taxonomy remains a topic of debate (El-Sherry *et al.*, 2015; Vrba & Pakandl, 2014). While mammalian coccidiosis is a notable problem, disease in chickens is commonly considered to be most significant (Blake *et al.*, 2020).

Coccidiosis in chickens can manifest as a haemorrhagic disease, usually associated with *E. tenella*, *E. necatrix* or *E. brunetti*, or malabsorptive disease caused by *E. maxima*, *E. acervulina*, *E. mitis* or *E. praecox* (Reid *et al.*, 2014). All seven species can be harmful, with *E. necatrix* and *E. tenella* considered to be most pathogenic (Long *et al.*, 1976; Williams *et al.*, 2009). *Eimeria acervulina*, *E. maxima* and *E. tenella* are usually most prevalent (Clark*, et al*., 2016; Hauck *et al.*, 2019; Haug *et al.*, 2008; Kumar *et al.*, 2014). Coccidiosis is most common when husbandry is poor, and is exacerbated by factors such as high stocking density, poor litter quality and high humidity.

**Impact**

*Eimeria* can affect chicken production and welfare. The nominal financial economic cost of coccidiosis in chickens was recently estimated to have exceeded UK£10.4 billion worldwide in 2016 (equivalent to ~ € 12 billion or US$ 14.5 billion), including costs of morbidity, mortality and control (Blake, *et al*., 2020). Morbidity, including poor body weight gain and food conversion ratios, represented the biggest cost component, varying from 51%-90% of the total cost between countries. The wider costs of *Eimeria* infection are difficult to quantify. *Eimeria* contribute to enteric dysbiosis, influencing microbiome diversity and structure (Macdonald *et al.*, 2017), and can also influence carriage of foodborne zoonotic pathogens including *Salmonella* Typhimurium and *Campylobacter jejuni* (Baba *et al.*, 1982; Macdonald *et al.*, 2019). *Eimeria* infection is well recognised as a major risk factor for necrotic enteritis, caused by *Clostridium perfringens*, independently estimated to cost the poultry production industry more than US$ 6 billion (Wade & Keyburn, 2015). The welfare consequences of coccidiosis are also considerable. In addition to the direct results of disease, the consequent diarrhoea reduces litter quality, contributing to footpad dermatitis and negatively impacting on overall welfare and technical performance (Abd El-Wahab *et al.*, 2012; de Jong *et al.*, 2014).

**Current approaches available for control of coccidiosis**

Modern production systems for chickens rely on control of *Eimeria*. Good husbandry is important, but frequently insufficient without routine chemoprophylaxis or vaccination. A range of sulphonamides were used as anticoccidial drugs from the 1940s, followed by the introduction of a series of synthetic and then ionophore products (Figure 1). Diclazuril was the last anticoccidial drug to be introduced in 1990, and no new active ingredients are currently close to market. Now, increasing public and legislative pressure is discouraging use of chemoprophylaxis in many production systems. Several anticoccidial drugs have been banned in areas such as the European Union (Squadrone *et al.*, 2008), and demand for ‘No antibiotics, ever’ products in the USA, where ionophores are regulated as antibiotics, is driving real reduction.

Alternative approaches to control *Eimeria* include use of live parasite vaccines (Figure 1). The first anticoccidial vaccines included live, fully virulent *Eimeria* oocysts representing one or more species(reviewed by Williams, 2002). The *Eimeria* used in these vaccines are considered ‘wild type’ (i.e. unmodified since collection from the field), although the long period of time since isolation for some has prompted use of the name ‘non-attenuated’, given the likelihood for divergence from modern field populations. Live vaccines are relatively complex, requiring multiple *Eimeria* species and, in some cases, strains because immunity induced by *Eimeria* infection is parasite species/strain-specific. Strain-specific immunity has been described for *E. acervulina*, *E. maxima*, *E. mitis* and *E. tenella* (Blake *et al.*, 2020), although only *E. maxima* has required more than one strain in some vaccine formulations. These wild-type or non-attenuated live vaccines can be highly effective and relatively cheap to produce, but risk compromising flock performance and occurrence of clinical disease if managed incorrectly (Blake *et al*., 2020; Shirley *et al.*, 2005). Non-attenuated vaccines are used widely across much of North America, as well as in parts of Africa and Asia. Recognising the risks posed by non-attenuated vaccines, a second generation of live-attenuated vaccines were first developed in the 1980s, using *Eimeria* lines modified by selection for precocious (i.e. abbreviated) development to attenuate pathogenicity while retaining immunogenicity (Shirley *et al*., 2005). Attenuated vaccines are used extensively in much of Europe, as well as parts of Africa, Asia and Australasia, primarily with layer and breeder stock owing to their relative cost and limited productive capacity (Blake *et al*., 2020).

Strategies for the use of live anticoccidial vaccines are evolving rapidly. Demand for ‘No antibiotics, ever’ poultry products in the USA is encouraging vaccination, using non-attenuated anticoccidial vaccines in place of chemoprophylaxis. The widespread occurrence of anticoccidial drug resistance is also driving demand for alternatives. Recent studies have demonstrated that multiple rounds of vaccination using drug susceptible live vaccine formulations can reduce drug resistance in *Eimeria* field populations, likely replacing or hybridising with resident strains and permitting a subsequent return to chemoprophylaxis (Chapman & Jeffers, 2015). The relative risk posed by use of non-attenuated vaccines can be managed using a bioshuttle approach, where vaccinated chicks receive grower and finisher diets containing anticoccidial drugs to limit parasite recycling and prevent vaccine-associated safety issues (Kimminau & Duong, 2019). Combined, these new management strategies to control coccidiosis have resulted in vaccination of ~35% of all broilers sold in the USA over the last five years (US industry data, unpublished). Previously, anticoccidial vaccine uptake was negligible in the US broiler sector, as remains the case in Europe today.

In addition to drugs and vaccines, a wide range of natural herbs and other botanicals or their extracts, organic acids, immunomodulators and complex carbohydrates, probiotics and prebiotics are now being developed to control coccidiosis (Khater *et al.*, 2020). Genetic mapping strategies are also being applied to inform selective breeding of elite commercial chicken lines towards improved resistance against infection and pathology (Boulton *et al.*, 2018; Hamzic *et al.*, 2015).

**Knowledge gaps, challenges and opportunities**

Access to scalable and cost-effective vaccines that can be deployed across different countries and poultry sectors remains a significant challenge. The recent dramatic increase in anticoccidial vaccine use in US broiler production has provided evidence that existing live vaccines can be scaled up and provide an alternative to anticoccidial drugs. The vaccines being used are non-attenuated and cost ~0.5 pence (UK) per dose. However, the live attenuated parasite lines used in European vaccines are considerably less fecund, hindering up-scaling, and currently cost between 3 and 8 pence per dose in the UK (Blake *et al*., 2020). Disruption in the supply of specific pathogen free chickens used in vaccine production can also create bottlenecks in vaccine availability. In the absence of change to regulatory and consumer requirements, alternatives are still required. Considerable progress has been made towards identification of immunoprotective antigens that can be used in recombinant or vectored vaccines, but none has made it to market (Blake *et al.*, 2017). One major constraint in deployment of such antigen-specific vaccines is an appropriate and effective delivery system. A range of possible vectors for oral administration, including *Bacillus*, *Salmonella*, transgenic *Eimeria* and yeasts such as *Saccharomyces cerevisiae*, are currently in development and could be appropriate (Hoelzer *et al.*, 2018; Pastor-Fernández *et al.*, 2020). Extension of these technologies to improve vaccines for turkeys will also be required. Establishment of effective oral vaccine vector systems can also support vaccination against other key pathogens of poultry.

Another major challenge remains our limited understanding of genetic diversity and population structure for *Eimeria*. Remarkably little is known about naturally existing diversity beyond a few well-established markers such as the rRNA genes and cytochrome C oxidase subunit I (COI) (Blake *et al.*, 2015). Improving understanding of existing genetic diversity and mechanisms of genome evolution may become fundamental to safeguard efficacy of the remaining anticoccidial drugs and future recombinant vaccines. New sequencing technologies, including the high-throughput Illumina platform and long read systems such as PacBio and Oxford Nanopore, are quickly becoming cheaper and more accessible. Application of these tools to *Eimeria* will improve our knowledge of species occurrence, diversity and evolution (Blake *et al*., 2015; Clark *et al*., 2016; Hauck *et al*., 2019; Hinsu *et al.*, 2018). The opacity of *Eimeria* population genetics has recently been emphasised by the characterisation of three new *Eimeria* species that infect chickens. Previously identified as operational taxonomic units (OTUs) x, y and z (Cantacessi *et al*., 2008), recent studies of morphology, pathology, genetics and phylogeny suggest these parasites warrant distinct species status and they have been named *Eimeria lata*, *Eimeria nagambie* and *Eimeria zaria* (Blake *et al*., 2021). It is notable that all three new species escape immune inhibition in chickens vaccinated with current anticoccidial vaccines from Europe and Australia, indicating a new coccidiosis risk as producers reduce their reliance on routine use of broad-spectrum anticoccidial drugs and rely much more on species-specific vaccines. The development of new and modified live parasite vaccines including these new species may therefore be required.

Most *Eimeria* are host and tissue specific in their development and this has hindered their study in *in vitro* systems (Marugan-Hernandez *et al.*, 2020), complicating fundamental biological as well as commercial applications. Primary cultures of avian cells have been used to propagate the *E. tenella* life cycle, but the efficiency of this is far too low for purposes such as vaccine production. Replication in non-avian cell lines fails to support completion of the full life cycle. As new technologies and cell lines become available *in vitro* culture is being re-investigated (e.g. Bussiere *et al.*, 2018). Improvements to *in vitro* culture offers opportunities to enhance biological understanding and could eventually contribute to vaccine production. Alongside this optimism, improved quantitative assays to measure parasite replication/gene expression and assess the longitudinal development of different life cycle stages *in vitro* offer opportunities to screen anticoccidial treatments before moving to studies in chickens (Marugan-Hernandez *et al*., 2020). New assays are also available for use *in vivo*, including sensitive species-specific quantitative PCR to measure parasite replication with higher throughput and greater reproducibility that traditional measures using microscopy (Nolan *et al.*, 2015). Development of a tool kit for transient and stable genetic modification of *Eimeria* has complemented these advances, supporting studies of fundamental biology and vaccine development (Pastor-Fernández *et al.*, 2019).

**Conclusions**

More than 72 billion chickens were produced globally in 2019, emphasising their major contribution to the production of protein for human consumption. Production systems for chickens are increasingly moving towards drug-free and/or extensive programmes in much of Europe and North America, while production is intensifying rapidly in many low- and middle-income countries. These changes are raising a series of new challenges to control of pathogens such as *Eimeria* species parasites. Recent developments in technical resources, public perceptions and legislative controls are changing our understanding of *Eimeria* and the ways in which we attempt to control them. It is likely that the coming decade will witness further significant changes, with new challenges and opportunities for innovation.

**References**

Abd El-Wahab, A., Visscher, C.F., Wolken, S., Reperant, J.M., Beineke, A., Beyerbach, M., et al. (2012). Foot-pad dermatitis and experimentally induced coccidiosis in young turkeys fed a diet without anticoccidia. *Poultry Science,* 91, 627-635. doi: 10.3382/ps.2011-01840

Baba, E., Fukata, T. & Arakawa, A. (1982). Establishment and persistence of *Salmonella* typhimurium infection stimulated by *Eimeria tenella* in chickens. *Research in Veterinary Science,* 33, 95-98.

Blake, D.P., Clark, E.L., Macdonald, S.E., Thenmozhi, V., Kundu, K., Garg, R., et al. (2015). Population, genetic, and antigenic diversity of the apicomplexan *Eimeria tenella* and their relevance to vaccine development. *Proceedings of the National Academy of Sciences of the United States of America,* 112, E5343-5350. doi: 10.1073/pnas.1506468112

Blake, D.P., Knox, J., Dehaeck, B., Huntington, B., Rathinam, T., Ravipati, V., et al. (2020). Re-calculating the cost of coccidiosis in chickens. *Veterinary Research,* 51, 115. doi: doi: 10.1186/s13567-020-00837-2

Blake, D.P., Pastor-Fernández, I., Nolan, M.J. & Tomley, F.M. (2017). Recombinant anticoccidial vaccines - a cup half full? *Infection, Genetics and Evolution,* 55, 358-365. doi: 10.1016/j.meegid.2017.10.009

Blake, D.P., Vrba, V., Xia, Jatau, I., Spiro, S., Nolan, M., et al. (2021). Genetic and biological characterisation of three cryptic *Eimeria* operational taxonomic units that infect chickens (*Gallus gallus domesticus*). *International Journal for Parasitology*.

Blake, D.P., Worthing, K. & Jenkins, M.C. (2020). Exploring *Eimeria* genomes to understand population biology: recent progress and future opportunities. *Genes (Basel),* 11. doi: 10.3390/genes11091103

Boulton, K., Nolan, M.J., Wu, Z., Psifidi, A., Riggio, V., Harman, K., et al. (2018). Phenotypic and genetic variation in the response of chickens to *Eimeria tenella* induced coccidiosis. *Genetics Selection Evolution,* 50, 63. doi: 10.1186/s12711-018-0433-7

Bussiere, F.I., Niepceron, A., Sausset, A., Esnault, E., Silvestre, A., Walker, R.A., et al. (2018). Establishment of an in vitro chicken epithelial cell line model to investigate *Eimeria tenella* gamete development. *Parasites & Vectors,* 11, 44. doi: 10.1186/s13071-018-2622-1

Cantacessi, C., Riddell, S., Morris, G.M., Doran, T., Woods, W.G., Otranto, D., et al. (2008). Genetic characterization of three unique operational taxonomic units of *Eimeria* from chickens in Australia based on nuclear spacer ribosomal DNA. *Veterinary Parasitology,* 152, 226-234. doi: 10.1016/j.vetpar.2007.12.028

Chapman, H.D. (1997). Biochemical, genetic and applied aspects of drug resistance in *Eimeria* parasites of the fowl. *Avian Pathology,* 26, 221-244.

Chapman, H.D. & Jeffers, T.K. (2015). Restoration of sensitivity to salinomycin in *Eimeria* following 5 flocks of broiler chickens reared in floor-pens using drug programs and vaccination to control coccidiosis. *Poultry Science,* 94, 943-946. doi: 10.3382/ps/pev077

Clark, E.L., Macdonald, S.E., Thenmozhi, V., Kundu, K., Garg, R., Kumar, S., et al. (2016). Cryptic *Eimeria* genotypes are common across the southern but not northern hemisphere. *International Journal for Parasitology,* 46, 537-544. doi: 10.1016/j.ijpara.2016.05.006

de Jong, I., Gunnink, H. & van Harn, J. (2014). Wet litter not only induces footpad dermatitis but also reduces overall welfare, technical performance, and carcass yield in broiler chickens. *Journal of Applied Poultry Research,* 23, 51-58.

El-Sherry, S., Ogedengbe, M.E., Hafeez, M.A., Sayf-Al-Din, M., Gad, N. & Barta, J.R. (2015). Sequence-based genotyping clarifies conflicting historical morphometric and biological data for 5 *Eimeria* species infecting turkeys. *Poultry Science,* 94, 262-272. doi: 10.3382/ps/peu007

FAOSTAT. (2021). Food and Agriculture Organization of the United Nations FAOSTAT database. Retrieved 4th February 2021, from http://faostat3.fao.org/home/

Hamzic, E., Buitenhuis, B., Herault, F., Hawken, R., Abrahamsen, M.S., Servin, B., et al. (2015). Genome-wide association study and biological pathway analysis of the *Eimeria maxima* response in broilers. *Genetics Selection Evolution,* 47, 91. doi: 10.1186/s12711-015-0170-0

Hauck, R., Carrisosa, M., McCrea, B.A., Dormitorio, T. & Macklin, K.S. (2019). Evaluation of next-generation amplicon sequencing to identify *Eimeria* spp. of chickens. *Avian Diseases,* 63, 577-583. doi: 10.1637/aviandiseases-D-19-00104

Haug, A., Gjevre, A.G., Thebo, P., Mattsson, J.G. & Kaldhusdal, M. (2008). Coccidial infections in commercial broilers: epidemiological aspects and comparison of *Eimeria* species identification by morphometric and polymerase chain reaction techniques. *Avian Pathology,* 37, 161-170.

Hinsu, A.T., Thakkar, J.R., Koringa, P.G., Vrba, V., Jakhesara, S.J., Psifidi, A., et al. (2018). Illumina Next Generation Sequencing for the Analysis of *Eimeria* Populations in Commercial Broilers and Indigenous Chickens. *Frontiers in Veterinary Science,* 5, 176. doi: 10.3389/fvets.2018.00176

Hoelzer, K., Bielke, L., Blake, D.P., Cox, E., Cutting, S.M., Devriendt, B., et al. (2018). Vaccines as alternatives to antibiotics for food producing animals. Part 2: new approaches and potential solutions. *Veterinary Research,* 49, 70. doi: 10.1186/s13567-018-0561-7

Khater, H.F., Ziam, H., Abbas, A., Abbas, R., Raza, M., Hussain, K., et al. (2020). Avian coccidiosis: recent advances in alternative control strategies and vaccine development. *Agrobiological Records,* 1, 11-25.

Kimminau, E.A. & Duong, T.T. (2019). Longitudinal response of commercial broiler operations to bio-shuttle administration. *Journal of Applied Poultry Research,* 28, 1389-1397.

Kumar, S., Garg, R., Moftah, A., Clark, E.L., Macdonald, S.E., Chaudhry, A.S., et al. (2014). An optimised protocol for molecular identification of *Eimeria* from chickens. *Veterinary Parasitology,* 199, 24-31. doi: 10.1016/j.vetpar.2013.09.026

Long, P.L., Joyner, L., Millard, B. & Norton, C. (1976). A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Folia Veterinaria Latina,* 6, 201-217.

Macdonald, S.E., Nolan, M.J., Harman, K., Boulton, K., Hume, D.A., Tomley, F.M., et al. (2017). Effects of *Eimeria tenella* infection on chicken caecal microbiome diversity, exploring variation associated with severity of pathology. *PLoS One,* 12, e0184890. doi: 10.1371/journal.pone.0184890

Macdonald, S.E., van Diemen, P.M., Martineau, H., Stevens, M.P., Tomley, F.M., Stabler, R.A., et al. (2019). Impact of *Eimeria tenella* coinfection on *Campylobacter jejuni* colonization of the chicken. *Infection & Immunity,* 87. doi: 10.1128/IAI.00772-18

Marugan-Hernandez, V., Jeremiah, G., Aguiar-Martins, K., Burrell, A., Vaughan, S., Xia, D., et al. (2020). The growth of *Eimeria tenella*: characterization and application of quantitative methods to assess sporozoite invasion and endogenous development in cell culture. *Frontiers in Cellular and Infection Microbiology,* 10, 579833. doi: 10.3389/fcimb.2020.579833

Nolan, M.J., Tomley, F.M., Kaiser, P. & Blake, D.P. (2015). Quantitative real-time PCR (qPCR) for *Eimeria tenella* replication - Implications for experimental refinement and animal welfare. *Parasitology International,* 64, 464-470. doi: 10.1016/j.parint.2015.06.010

Pastor-Fernández, I., Kim, S., Marugan-Hernandez, V., Soutter, F., Tomley, F.M. & Blake, D.P. (2020). Vaccination with transgenic *Eimeria tenella* expressing *Eimeria maxima* AMA1 and IMP1 confers partial protection against high-level *E. maxima* challenge in a broiler model of coccidiosis. *Parasites & Vectors,* 13, 343. doi: 10.1186/s13071-020-04210-2

Pastor-Fernández, I., Pegg, E., Macdonald, S.E., Tomley, F.M., Blake, D.P. & Marugan-Hernandez, V. (2019). Laboratory growth and genetic manipulation of *Eimeria tenella*. *Current Protocols in Microbiology,* 53, e81. doi: 10.1002/cpmc.81

Reid, A.J., Blake, D., Ansari, H., Billington, K., Browne, H., Dunn, M., et al. (2014). Genomic analysis of the causative agents of coccidiosis in domestic chickens. *Genome Research,* 24, 1676-1685.

Reid, W.M. (1990). History of avian medicine in the United States. X. Control of coccidiosis. *Avian Diseases,* 34, 509-525.

Shirley, M.W., Smith, A.L. & Tomley, F.M. (2005). The biology of avian *Eimeria* with an emphasis on their control by vaccination. *Advances in Parasitology,* 60, 285-330.

Squadrone, S., Mauro, C., Ferro, G.L., Amato, G. & Abete, M.C. (2008). Determination of amprolium in feed by a liquid chromatography-mass spectrometry method. *Journal of Pharmaceutical and Biomedical Analysis,* 48, 1457-1461. doi: 10.1016/j.jpba.2008.09.024

Vrba, V. & Pakandl, M. (2014). Coccidia of turkey: from isolation, characterisation and comparison to molecular phylogeny and molecular diagnostics. *International Journal for Parasitology,* 44, 985-1000. doi: 10.1016/j.ijpara.2014.06.004

Vrba, V. & Pakandl, M. (2015). Host specificity of turkey and chicken *Eimeria*: controlled cross-transmission studies and a phylogenetic view. *Veterinary Parasitology,* 208, 118-124. doi: 10.1016/j.vetpar.2015.01.017

Wade, B. & Keyburn, A. (2015). The true cost of necrotic enteritis. *World Poultry,* 31, 16-17.

Wierup, M. (2001). The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. *Microbial Drug Resistance,* 7, 183-190. doi: 10.1089/10766290152045066

Williams, R.B. (2002). Fifty years of anticoccidial vaccines for poultry (1952-2002). *Avian Diseases,* 46, 775-802.

Williams, R.B., Marshall, R.N., Pages, M., Dardi, M. & del Cacho, E. (2009). Pathogenesis of *Eimeria praecox* in chickens: virulence of field strains compared with laboratory strains of *E. praecox* and *Eimeria acervulina*. *Avian Pathology,* 38, 359-366. doi: 10.1080/03079450903186028

**Figure legends**

**Figure 1.** Expansion of global chicken production and products used to control *Eimeria*. Figure adapted from Reid (1990). The number of chickens produced each year downloaded from FAOSTAT (2021). The coloured boxes indicate the periods when new sulphonamide (orange), synthetic (green) and ionophore (red) drugs were introduced to control *Eimeria* in chickens (Chapman, 1997; Reid, 1990). The hollow box indicates the period during which no new anticoccidial drugs have been introduced. Blue arrows indicate the introduction of the first non-attenuated (wild-type) and attenuated anticoccidial vaccines. \*Introduction of a ban on antimicrobial growth promoters in Sweden (Wierup, 2001). #Introduction of a ban on anticoccidial drugs such as amprolium as prophylactic food additives in the European Union (Squadrone *et al*., 2008).