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Review Article

DIAGNOSTIC METHODS OF ENTEROHAEMORRHAGIC *ESCHERICHIA COLI* IN HUMAN MEDICINE AND BENINESE CURRENT SITUATION ON THE PATHOVAR

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ABSTRACT
Enterohaemorrhagic *Escherichia coli* infections are becoming more frequent. They sometimes manifest as epidemics leading to complications of varying severity and are often unpredictable. Recognition of this disease based on clinical, epidemiological and biological criteria will allow rapid and adequate management of infected patients. The purpose of this article is to synthesize data on the different methods of diagnosing EHEC infections, with particular attention to strains responsible for human infections. We put special emphasis on the current state of knowledge of pathovars in Benin after a brief review of African situation. This synthesis made it possible to note the existence of a multitude of methods of diagnosis of EHEC. Although some of these methods are routinely accessible, they sometimes require the use of methods reserved to specialized laboratory for epidemiological studies. In addition, the presence of EHEC in Africa is well established despite the relatively lower detection rate. Benin is now on the list through these recent findings on the existence of pathovar in the country.

KEY-WORDS: enterohaemorrhagic *E. coli*, diagnostic methods, human infections.

INTRODUCTION

Shiga toxins producing *E. coli* (STEC) are recognized internationally as emerging pathogens. The name STEC includes all *E. coli* strains possessing the stx genes encoding a particular toxin called Shigatoxin. They are associated with food epidemics sometimes large-scale and often very serious. Although not all STECs are pathogenic for humans, some strains called enterohaemorrhagic *E. coli* (EHEC) are responsible for serious human infections. Human involvement results in the development of haemorrhagic colitis and / or hemolytic and uremic syndromes (HUS) that can lead to potentially fatal kidney sequelae (Bryan *et al*., 2015), requiring long-term treatment, especially in children under 3 years old. People over 60 are also considered as member of population at risk (EFSA, ECDC, 2015). These infections constitute a major problem in public health because of the extreme severity of the clinical manifestations. Although the most frequently encountered EHEC strains in outbreaks are serotype O157: H7, many other serotypes such as O26: H11, O103: H2, O111: H8 or O145: H28 have also been implicated in epidemic or non-epidemic infections (AFSSA, 2010, Hussein, 2007). But there are many other STEC serotypes that are more rarely involved in human cases or epidemics. This was the case very recently of serotype O104: H4, responsible for two epidemics in Germany and France. The main natural reservoir of STEC is the digestive tract of cattle, although a carry of STEC in sheep and goats has been reported. Contamination of foods derived from ruminants is the major cause of human infections. These are ground beef, undercooked vegetables, raw and badly washed vegetables, and raw milk products.

Since 1982, STECs have been responsible for many cases of human infections around the world, mainly as a result of the consumption of contaminated food of animal origin. Thus, several epidemics, associated with STEC, have been identified in France, such as the 2005 epidemic involving raw milk camembert containing STEC O26: H11, then more recently also in 2011 (sprouted seeds, STEC O104: H4). , 2012 (beef, STEC O157: H7) and 2013 (raw milk cheese, STEC O157: H7). This is also the case in Africa where epidemics have been recorded in several regions (Effler *et al*., 2001, Cunin *et al*., 1999 and Koyange *et al*., 2004). These different epidemics point not only to the need to better understand the behavior of STECs and to be able to detect them in food matrices, but also to be able to diagnose these infections in human medicine. The present work proposes, through a bibliographical synthesis, to present the clinical diagnosis and the laboratory diagnosis of EHEC infections, in order to constitute a support to the Beninese researchers for more research in the field.

Methods

This review was done using a critical analysis of the scientific literature. The information was collected by querying the Medline, Elsevier and Google scholar bibliographic databases. The search was limited to documents, theses and specific publications in French or English language. A synthesis has been made and the results are presented in the following lines.

Clinical diagnosis of EHEC

Clinical diagnosis is based on an evaluation of the epidemiological criteria, the manifestations and symptoms of the disease, the evolution of the disease and the nature of the intestinal evacuations. Anyone who has ingested an EHEC strain may develop symptoms, although children under 5 and people over 60 are more susceptible and more severely affected by these infections (Karmali *et al*., 2010). Many factors can influence this sensitivity: health status, the number of Stx toxin receptors and the medical treatments followed including antibiotics. Thus, an EHEC infection can take various forms, from asymptomatic carriage to subject death, to potential systemic complications such as hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP).

Colitis and bleeding colitis

The most common form of EHEC infection is the appearance of watery diarrhea that may progress to haemorrhagic colitis in generally afebrile or subfebrile individuals (Griffin, Tauxe, 1991). Heamorrhagic colitis is observed in 90% of patients diagnosed with EHEC positive (Tarr *et al*., 1996). Incubation period, which is longer than that observed for other infectious diarrhea, is between 2 and 10 days (Griffin, Tauxe, 1991). Symptoms resolve spontaneously in a few days in 90% of cases. But for 10% of them complications appear such as HUS or PTT and in very rare cases, narrowing of the intestine (Tarr *et al*., 2005).

Hemolytic Uremic Syndrome (HUS)

Hemolytic and Uremic Syndrome is defined by the association of microangiopathic hemolytic anemia (MAT) with the presence of fragmented red blood cells (schizocytes), thrombocytopenia and acute renal failure. It corresponds to MAT lesions affecting the kidneys and possibly other viscera, characterized by a thickening of the walls of the glomerular capillaries and / or arterioles, and the presence of platelet micro aggregates in the capillaries and arterioles (Mariani-Kurkdjian and Bonacorsi, 2014). It usually occurs after bloody prodromal diarrhea within 7 to 15 days of ingestion. The incidence of HUS is 10% in children under 10 years of age and 10% to 20% in the elderly (Griffin, Tauxe, 1991). It is the cause of the leading cause of kidney failure in infants. Other organs such as the pancreas, liver and central nervous system can also be affected. Central nervous system involvement appears to be the major cause of death in subjects (Decludt *et al*., 2000, Loirat *et al*., 1992). This clinical picture is characteristic and does not generally pose a diagnostic problem. The current treatment of this pathology remains today mainly symptomatic.

Thrombotic thrombocytopenic purpura (TPP)

More commonly seen in adults, it is exceptional in children and the elderly and usually lasts a few days to a few weeks. It is a syndrome characterized by the appearance of neurological signs in the form of microangiopathic and thrombotic hemolytic anemias associated with onset of fever and renal dysfunction (Tarr *et al*., 2005). It remains rare after an EHEC infection. After clinical suspicion, the diagnosis is based on the identification of the pathogen in the laboratory.

EHEC Diagnosis in laboratory

Many methods of diagnosing EHEC infections are used. These are the detection of toxins on cell lines, genetic methods, biochemical methods, immunological methods and serodiagnosis. Some of them are routinely accessible; others are reserved for specialized laboratories.

The diagnosis is generally based on the detection directly in the stool and / or after culture of the main virulence genes of EHEC on the one hand, and on the increase of the serum titer of specific anti-lipopolysaccharide antibodies on the other hand. Part (anti-LPS) (EFSA 2013, Espie *et al*., 2008, Gouali *et al*., 2013) (Figure 1). Whatever the diagnostic method used, the quality of the result depends on the quality of the sample to be treated. Thus, sampling is the first important step in the pathogen's research protocol.

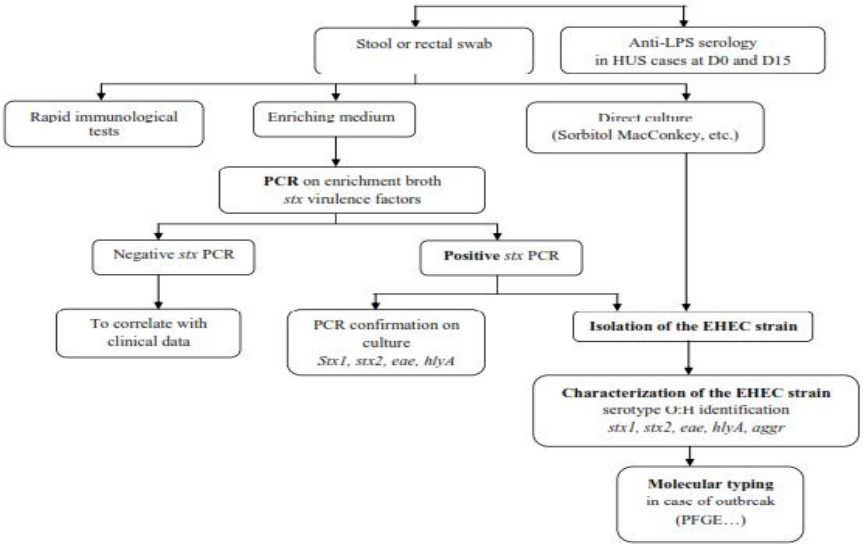

FIGURE 1. Diagnosis of EHEC infections (Espié et al., 2008), PFGE: Pulsed-Field Gel Electrophoresis

EHEC infections diagnosis is difficult, as these bacteria are rapidly eliminated from the digestive tract. The amount present in the stool is very small (<10 2 CFU / g stool), especially at the time of the HUS where stool collection should take place at maximum 4 to 6 days after the start of the digestive prodromes so that the analysis is contributive

(Tarr *et al*., 1990). Patients admitted to SHU often have intestinal transit stopping, stool can also be collected by rectal swabbing (Mariani-Kurkdjian and Bonacorsi, 2014). In addition, the sample must be taken before taking any antibiotic and must be transported quickly to the laboratory, or kept at + 4°C and sent to a transport medium if the analysis is not carried out on site (Mariani). Kurkdjian and Bonacorsi, 2014).

Detection of toxins on cell lines

Toxins detection is done through the cytopathogenic effect on a Vero or Hela cell. This is indeed the reference technique for the search for Stx free toxins in stool or on isolated strains. Such a test is specific but difficult to implement. It can only be done in a specialized laboratory (AFSSA, 2003). In the case of a mixture of faeces, it is desirable to improve the sensitivity of the test by treatment with polymyxin B or mytomycin; which releases the toxin bound to the cells. In the absence of cell cultures, other methods can be used to detect verotoxin production, such as ELISA or agglutination, and PCR can detect vt genes.

Genetic Methods

These methods consist in the detection of stx genes encoding verotoxins, either directly on the total genome (hybridization of DNA probes), or after amplification of part of the desired genes (PCR). Considering the very small amount of EHEC present in the stool, gene amplification in situ by PCR of the genes coding for Stx1 and Stx2 and / or the eae gene in the stool represents a method of choice for diagnosis. It is the most sensitive method for detecting EHEC from feces, usually after enrichment for 4 to 6 hours in peptone water (Mariani-Kurkdjian and Bonacorsi, 2014). The broth is subcultured on selective agar. The PCR can be carried out directly on the broth and / or on the layer of bacterial colonies having grown on the selective medium. However, it can currently only be performed by specialized laboratories. Numerous PCR systems have been described. The primer system developed by Lin et al. (1993) can detect, in a single system, all known variants of stx genes. Other virulence gene detection systems can be searched for in combination in multiplex PCR systems. In addition, real-time PCR methods allow faster diagnosis than conventional PCR methods (EFSA 2013, Gouali 2013). When the PCR is carried out directly on the enrichment broth, a positive PCR response requires the isolation of the bacterium in question which is indispensable for the characterization of the pathogenicity factors (stx, eae, ehxA) (EFSA, 2013), in particular stx variants considered as a predictor of the severity of EHEC infections (Orth et al., 2007). However, isolation of the strain is sometimes very difficult and involves many biochemical tests.

Phenotypic methods

Isolation of strains

Most of the biochemical reactions of STEC are typical of *E. coli*, and respond to the IMVIC test (Indole-Red Methyl-Voges-Proskauer, Citrate) which differentiates them from other Enterobacteriaceae, with the exception of *E. coli* O157: H7 which has the particularity of not fermenting sorbitol and to present a negative β-glucuronidase activity in most cases.

The isolation of the strain is sometimes very difficult because of the sometimes very small amount of EHEC in the stool. Thus, in human medicine, it is conventional to use buffered peptone water, supplemented with vancomycin. After this enrichment phase of 4 to 6 hours, the stool is cultured on specific media revealing the biochemical properties of *E. coli* O157: H7 (Brugere *et al*., 2013) above-mentioned. The dedicated media are Mac Conkey's Sorbitol-CT(Cefixime-Tellurite) medium (Karmali *et al*., 1993, Brugere *et al*., 2013), RAPID'E agar. coli O157: H7. Chromogenic media for the detection of O157 strains have also been developed, such as CHROMagar O157 medium, ChromID O157: H7 medium.

Moreover, the non-O157 EHEC strains have no common biochemical property allowing their detection on a particular medium. Traditional media are used for enteropathogenic bacteria such as Drigalski, Hektoen.

An alternative solution for the isolation of EHEC strains is the use of enterohemolysin agar. The method is based on the fact that a large proportion of EHECs have the property of producing an agar-detectable enterohemolysin containing washed sheep erythrocytes, supplemented with Ca2 + ions (Beutin *et al*., 1989). However, some non-O157 STEC O157 and sorbitol-fermenting STEC O157 may not produce enterohemolysin and are therefore not detected on blood agar (Bielaszewska *et al*., 1998).

In addition, the presence of a large number of non-STEC strains producing haemolysin may interfere with the identification of suspect colonies on blood agar. Possé et al. (2008) developed a protocol to isolate and detect the other four serogroups (O26, O103, O111, and O145). After appropriate enrichment, the colonies are isolated on agar plates containing, inter alia, antibiotics and a substrate (X-gal). These different components make it possible to phenotypically differentiate non-O157 STECs. Another agar, Rainbow O157 agar, makes it possible to identify the different serogroups of STEC: O157, O26, O103, O111, O145, O45, O121.

Characterization of strains

It usually starts with the identification of serotype O: H. Serotyping O consists of agglutination of colonies using anti-O specific sera (directed against LPS), making it possible to detect certain serotypes known to be EHECs (O157, O26, O111, O55, O145, *etc*.).). The search for virulence genes must be carried out in case of a positive reaction for one of the serotypes. Serotyping H can be performed secondarily. It is essential for epidemiological studies (EFSA 2013, Gouali et al., 2013). For more rare serotypes that require rare typing sera or in case of auto-agglutinable strains, molecular techniques (PCR, RFLP and sequencing) can be used to determine the "molecular serotype".

Immunological methods

The detection of EHEC strains directly in the stool or after an enrichment phase in broth can also be done through various immunological tests (Table 1):

- EIA (Enzyme Immuno-Assay) tests,
- OIA (Optical Immuno-Assay),
- Immunochromatography, etc.

These highly specific and sensitive immunoassays detect O157 antigen and / or Stx toxins. Very easy to implement, they must be used according to the strict recommendations of the manufacturers. In addition, they are an alert for the clinician when they are positive. However, although having good sensitivity and specificity, their reading is

sometimes difficult and they can give false positive results by cross-reactions with enteric viruses or other bacteria. Thus, they must always be confirmed by molecular methods (EFSA, 2013).

TABLE 1. Immunological tests for the détection of EHEC strains in stool or enrichment broth (Mariani-Kurkdjian et Bonacorsi, 2014)

Test	Laboratory	Méthods	Target	Specimen (*)
BioStar OIA SHIGATOX	Inverness Medical	OIA (Optical ImmunoAssay)	Stx toxins (without distinction Stx1 and 2)	Stools Enrichment broth
Duopath® Verotoxins GLISA test	Merck	GLISA (Gold Labelled ImmunoSorbent Assay)	Stx1 and Stx2	Stools Enrichment broth
ImmunoCard STAT!® EHEC	Meridian Biosciences	Immunochromatography	Stx1 and Stx2	Enrichment broth
RIDA®QUICK Verotoxin / O157 Combi	R-Biopharm	Immunochromatography	Antigen O157 Stx toxins (without distinction Stx1 and 2)	Enrichment broth
SHIGA TOXIN QUIK CHEK	Alere	Immunochromatography	Stx1 and Stx2	Stools Enrichment broth

(*)Possible use from strains for BioStar OIA SHIGATOX and Duopath® Verotoxins GLISA tests

Serodiagnosis

This diagnosis is useful in patients who have had HUS and whose EHEC stool test was negative at the time of HUS. It consists in highlighting antibodies directed against the lipopolysaccharide of the bacterium. The search for verotoxigenic antibodies is not carried out because they are not very immunogenic, few patients develop antibodies directed against verotoxins. Generally, during STEC infections, patients develop anti-lipopolysaccharide antibodies (IgG, IgM and IgA) within 7 to 10 days. These antibodies are detectable at often very high levels, even several weeks after the onset of digestive prodromes (Bitzan *et al*., 1991). Serologic diagnosis should be made on "early" serum and "late" serum, usually 2-3 weeks after the first, in order to look for an increase in the titre of antibodies to the infection. However, a high titre, even on a single serum, can sometimes be a reliable indicator of a recent *E. coli* O157 infection. Currently, the detection of O157 serogroup LPS antibodies, as well as other serogroups (O2, O91, O103, O111, O128, and O145), can be carried out by various techniques: ELISA, Western-blot, immunoblotting or indirect haemagglutination (Chart, 1993, Paton, Paton, 1998). The search for these antibodies is essential for the diagnosis and for epidemiological studies when the direct detection of the genes coding for Stx toxins and / or EHEC in the stool is negative, or could not be performed (Espié *et al.*, 2008).

***E. coli* Shiga toxin producers: Inventory in Benin**

It is important to recall the general situation of Africa on this pathovar before addressing the specific case of Benin. Thus, the revelation of EHEC /STEC being related to the technical capacity of laboratories to detect them, in a given environment, it is obvious that the real geographical extension of these agents remains to be elucidated. In Africa, EHEC / STEC are seldom routinely sought in view of the modest biological diagnostic capabilities of several laboratories (Wittenberg 1999, Hiko *et al*. Thus, several infections remain unidentified (Wittenberg, 1999, WHO, 2005), or STEC infections are attributed to Shigella (Aragon *et al*., 1993, Malakooti *et al*., 1997), which clinically causes a similar syndrome. STEC, including diarrhea. However, STEC /EHEC have already been demonstrated in more than twenty African countries in several studies, and have been responsible for a dozen serious epidemics. They have also been implicated in several cases of infectious diarrhea, particularly in children (Dadié *et al*., 2013). The first data on the *E. coli* pathovar and other shiga toxin producing serovars of the enterohaemorrhagic *E. coli* class appeared from 1990 (Browning *et al*., 1990). But STEC were considered a real public health problem in Africa with the outbreak of the epidemic in Swaziland, Mpumalanga and KwaZulu-Natal (Isaäcson *et al*., 1993, Effler *et al*., 2001). Several regions of the continent, from North to South, from East to West, are concerned by STEC / EHEC infections. However, STECs have not yet been detected in some African countries despite the fact that research is being conducted, or data is not available for some countries such as Ghana, Somalia, Djibouti, Sudan, Mozambique, Gabon, Mali and Mauritania (Dadié *et al*., 2013). This finding shows, however, that many African countries are aware of the risk of infection with this pathovar. In countries where STEC have been isolated in Africa, apart from the cattle sector, which is an important reservoir for pathovars, other STEC vehicles have been reported, including sheep and goats (Hiko *et al*., 2008), sheep, carcass and fecal matter (Chahed, 2007). Moreover, water alone is considered as a risk factor for the emergence of STECs in the continent (Effler *et al*., 2001, Obi *et al*., 2004). It is simply deduced that the main route of transmission in humans is the food route, which, moreover, has been frequently mentioned in other continents. Person-to-person transmission is not common but has been highlighted in some countries including Nigeria (Okeke *et al*., 2003).

In addition, different methods were used for the detection of STEC / EHEC. They are conventional or standardized. The isolation on selective medium for *E. coli* O157: H7 has been carried out by several authors: SMAC or SMAC-CT (Cunin *et al*., 1999, Tuyet *et al*., 2006), Chromogenic-Agar (Dadié *et al*., 2000, Muller *et al*., 2001), or on media for Mc Conkeyenterobacteria, BCP, MUG-Agar, petrifilm (Al-Gallas *et al*., 2007, Chahed, 2007). It is generally followed by identification, by the determination of biochemical characters (Dadié *et al*., 2000, Cohen *et al*., 2008, Badri *et al*., 2009). The characterization of the virulence factors by research has been carried out, for several studies by PCR, and also by probe or genetic hybridization (Kaddu-Mulindwa *et al*., 2001, Valentiner-

Branth *et al*., 2003, Okeke *et al*., 2003). In other cases, isolation on selective media is combined with ELISA and immunomagnetic techniques for the isolation of strains in water (Muller *et al*., 2003). The in vitro culture technique for determining the pathogenicity by testing Vero permissive cells or the phenotypic nature of strains has been performed in several studies. Some typing or subtyping methods (IMS, VIDAS, PCR-RFLP, PFGE) are very little used, because they require the intervention of specialized laboratories in Africa or are the responsibility of an expertise, most often outside the continent (Dadié *et al*., 2013). In Benin, few studies have been carried out to search for enterohaemorrhagic *Escherichia coli* strains in both the medical and food fields. Since *E. coli* is part of the human commensal flora and therefore rarely pathogenic, stool cultures are often declared negative when the bacterium involved is *Escherichia coli*. However, strains of *E. coli* can be pathogenic and cause infections such as gastroenteritis, urinary tract infections, meningitis or sepsis (Dembélé *et al*., 2015).

Until 2013, the situation in Benin regarding *Escherichia coli* O157 and *E. coli* not O157 producers of shigatoxins was not known. *E. coli* O157 was isolated for the first time by Bankolé *et al.* (2014) during a study on the evaluation of the contamination of some food products by this pathovar in Benin. The bacterium was isolated with a prevalence of 5.26% of livestock samples from farms in the south of the country. In addition, *E. coli* O157 was found in several other food products in the same study. These include pork, with a contamination rate of 11.11%, 25% vegetable leaves and 8.3% core samples. New data on the possible virulence of *E. coli* strains isolated from food will appear two years later with the work of Moussé *et al*. (2016). By analyzing a set of 216 food samples, these authors detected strains of *Escherichia coli* producing shiga-toxins stx1 (4.35%) and stx2 (47.83%). These food products consisted of street food (Russian salad, vegetable sauce and cooked rice) collected in two main cities in southern Benin. In addition to food products *E. coli* O157 was found at a rate of 16.7% in irrigation water samples (Bankolé *et al*., 2014).

The different methods used in the studies undertaken in the context of the detection of STEC in Benin show the isolation on media selective for *E. coli* O157, Mac ConkeySorbiotl, CHROMagar O157 (Bankolé *et al*., 2014), Rapid ' *E. coli* (Moussé *et al*., 2016). In some cases, sample processing included a pre-enrichment phase, an enrichment phase, an isolation phase, a purification phase, a biochemical identification phase, and a serological identification phase. Moreover, the characterization of strains isolated by the search for virulence genes was done by PCR by Moussé *et al*., who targeted mainly three genes (vt, stx1, stx2). In view of the results of the work available, it is known that *E. coli* O157 has been isolated in several food products. But to date, no epidemic or sporadic cases have been reported in the country. However, potential risk factors exist in the Beninese environment that may favor the occurrence and development of STEC. These include precarious hygiene in a number of localities in the practice of cattle breeding, and a failing health system. As humans become contaminated by the consumption of water and contaminated food such as undercooked beef, unpasteurized milk and dairy products, the risk of intoxication of the population exists. Therefore, better knowledge of epidemiology, research methods and the availability of detection tools and techniques is essential.

CONCLUSION

Enterohaemorrhagic *E. coli* are emerging pathogens responsible for food borne illnesses that can cause serious pathologies. Since their worldwide spread is a reality, it is important to master their diagnosis in human medicine in order to allow proper management of infected patients.

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