

Distribution, Virulence and Diversity of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* at Major Brassica Growing Areas of Ethiopia

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ABSTRACT

A study was conducted to investigate the virulence and diversity of blackleg in major brassica growing areas of Ethiopia. The highest blackleg severity was recorded at Holeta on station canola type cultivar with severity range of 3.5% to 25.6%. The rest visited fields were free from black leg disease. Most of the fields were found covered with *Brassica carinata* which is under species has BB genome which confers resistance to blackleg. A total of 48 fungal isolates were recovered from leaves and stem of *Brassica* species. 52% of the isolate goes to *L. biglobosa* followed by *L. maculans* 31.25%. Morphological characteristics of the isolates were studied on a PDA medium at $25 \pm 1^\circ\text{C}$: Colonies were found circular in shape after 5 days, and were observed in isolates: BLHH-1, BLHH-2, BLHH-3, BHLL-4, LM-1, LM-2, LB-1, and LB-2. Mycelia were loose, colored white to white smoke. Some of them form colonies with irregular round shape and lobular edges. The pycnidia of the fungus were black, globose to subglobose in shape, the single-celled conidia, hyaline and fusiform with diameters of $4-5 \times 1.5-2 \mu\text{m}$. From the result slow growth was observed on *L. maculans* isolate with high sporulation, whereas faster growth rate was observed on *L. biglobosa* with low sporulation. For the purpose of isolates separation, based on pigment formation on liquid Czapek agar, it was observed that after 30 days isolates LM-1, LM-2, LB-1 and LB-2 where produce yellow-brown pigment which indicate places isolates in a group of non-aggressive strains in conformity with the *L. biglobosa*. Isolate BLHH-1, BLHH-2, BLHH-3 and BLHH-4 which did not produce pigment; the situation indicates the aggressiveness of the isolate and which is under group *L. maculans*. Blackleg were found less distributed in major growing areas of Ethiopia, however *L. maculans* and *L. biglobosa* were confirmed their presence based on morphological and cultural characteristics. So in line with resistance variety development other management options need to be address to make rapeseed return to production.

Keywords: Blackleg; *Leptosphaeria biglobosa*; *Leptosphaeria maculans*; Morphological characteristics

INTRODUCTION

Ethiopia is the country with large diversity and history of using *Brassica* species as vegetable and oilseed. The research system start introducing *Brassica napus* in 1970 from Australia and Europe the variety is known for production of edible oil [1]. Since then production of the crop have been continued in Bale and Arsi zones large state farms till the black leg epidemics occur in 1983.

Effort in crop protection research on oilseed has been very low compared to that of vegetable, legumes and cereals, and the record showing diseases as being among production constraints in oil crop in Ethiopia [2]. Blackleg caused by the fungus *Leptosphaeria maculans* (anamorph *Phoma lingam*), is a devastating diseases present most of rapeseed growing areas. The pathogen can infect all part of the plant, but stem canker is the most serious symptom as it causes plant lodging and yield loss [3].

Protecting crops from catastrophic yield losses caused by plant pathogens is a major goal of agriculture, to safeguard global food

security in response to growing concerns about food shortages and climate change [4]. Among the bacterial, fungal, viral and phytoplasmic-like diseases, blackleg is the most important global disease of *B. napus* and causes annual yield losses of more than \$900 million in Europe, North America and Australia [5]. Under epiphytotic conditions, this disease can cause yield losses of up to 90 per cent [6], in Ethiopia due to blackleg epidemics in 1983 up to 100% yield loss were recorded and make the crop out of production [1]. Currently Ethiopia has emerged as one of the largest importer of edible oil in the world market and more than 85% of domestic demand is met through imports even if the country has the potential of producing this crop.

Epidemics of the disease initiated by air-borne *L. maculans* ascospores released from pseudothecia produced on stem debris of previous crops [7]. The symptomatology of the diseases starts with the appearance of gray-green to ash-gray lesions on the lower leaves, when the diseases advances basal stem cankers, small grey oval lesions on the leaf tissue and root rot were observed.

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Today is more than 37 years since the disease is reported from Ethiopia; however its distribution, current status and pathogen virulence and diversity have not yet known and determined. Since population of blackleg pathogen are complex of at least two genetically distinct groups, now referred to as two different species, *L. maculans* (highly virulent, A-group) and *L. biglobosa* (weakly virulent, B-group) [3] and according to the interaction phenotype (IP), isolates can be classified in to at least five pathogenicity groups (PGs): PG-1, PG-2, PG-3, PG-4 and PGT, there is no research done in Ethiopia to see the virulence and diversity of blackleg pathogen complex. The present study have been conducted to see the distribution and status of blackleg disease and to investigate the virulence and diversity of isolate obtaining from major growing areas.

MATERIALS AND METHODS

Description of the study area

Field survey and sample collection were done during the main season of 2018 major *brassica* growing areas of (North Shewa, South West Shewa and West Shewa and Gurage zone some places) of Ethiopia (Figure 1). The zone has a bimodal rainfall distribution and is a typical sub-humid, high altitude agro-climatic zone.

Assessment of Black leg disease

Purposive sampling was applied to select fields following the main roads and accessible routes. In the selected fields, black leg disease assessment was made along the two diagonals in an ("X" fashion). Up to five spots per field were assessed. The disease were rated using a 0–6 scoring system whereby ratings of 0, 1, 2, 3, 4 and 5 respectively are assigned to plants with 0, 1–5, 6–25, 26–50, 51–75 and 76–100% of the cross section infected, and a rating of 6 for dead plants. Disease incidence was determined as percentage of plants showing blackleg symptoms out of the plants sampled.

Data on geographical information (latitude, longitude and altitude) of each field was recorded using a GPS (Legend GPS system, Garmin). The GPS data (latitude and longitude) were used to plot surveyed fields on a map using computer programmed Arc View 3.0. During the survey, growers were asked information on locality name, field history, varieties grown, cropping systems, planting date, weeding practices and disease control practices employed to determine relationship with the disease. Stem and leaf samples showing typical symptoms of blackleg were collected.

Isolation of the pathogen

Samples of infected *brassica* plants with black leg symptom were

Black leg Disease Distribution Map in Ethiopia

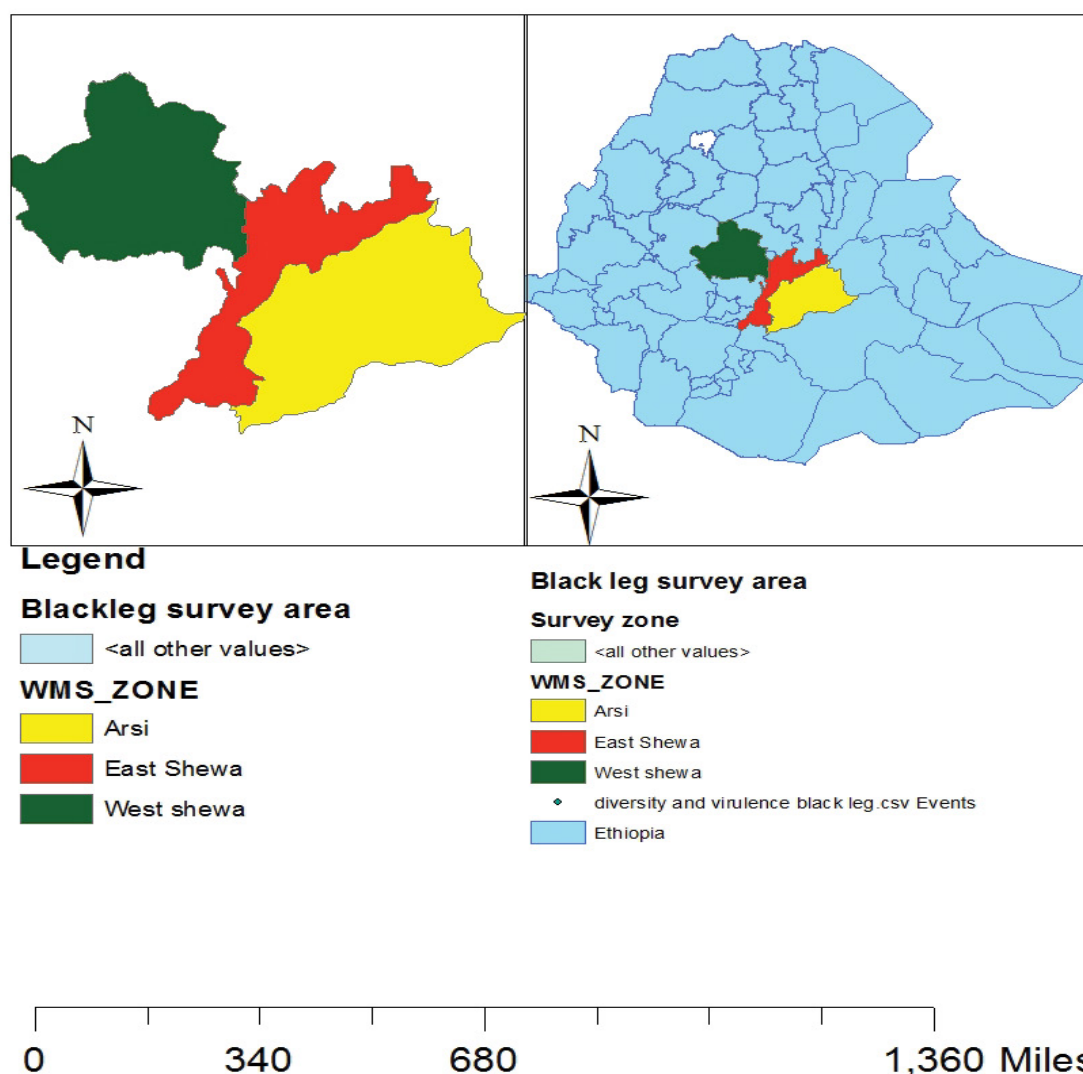


Figure 1: Map blackleg disease samples collected.

collected during the survey. They were analyzed in Holetta Agricultural Research Center pathology laboratory. Isolation was done from plant parts (root crown, stem, leaf,) with clear symptoms of the disease. Fragments of infested tissue were submerged in a 3% sodium hypochlorite solution for 3 to 5 minutes, washed with distilled water and dried naturally. After drying, fragments of infested tissue were placed on a Potato Dextrose Agar (PDA). Petri dishes with PDA were placed in incubator at $25 \pm 1^\circ\text{C}$.

Morphological characteristics

Macroscopic traits of fungal isolates (growth, appearance and color of mycelia, appearance of colony edges, the presence of fruiting bodies, and pigment secretion) were examined on a PDA at $25 \pm 1^\circ\text{C}$ (for all 8 isolates tested), as well as microscopic traits - mycelium texture, appearance, size, shape and color of pycnidiospores and pycnidia [8]. The trial was set up in four replications. Mycelia growth of the isolates was measured in cm on days 5, 10 and 15.

Pigment formation on a liquid Czapek agar plate

For the purpose of dividing isolates and affiliation to their respective groups of the tested fungi (*L. maculans* and *L. biglobosa*), pigment formation was analyzed on a liquid Czapek agar. Agar was placed into the test tubes (10 ml per tube) and sterilized in the autoclave at 120°C for 20 minutes. After cooling, fungal mycelium of all tested isolates was placed onto the agar and under the photoperiod of 12 h at 20°C . Pigment formation was monitored for 4 weeks, based on the changes in color of the agar.

RESULTS AND DISCUSSION

Disease distribution

During the survey, the highest blackleg severity was recorded at Holetta on station canola type cultivar with severity range of 3.5% to 25.6% (Table 1). The rest visited fields were free from black leg disease. During the survey most of the fields were covered with *Brassica carinata* variety (Yellow Dodola) which is under species has BB genome which confers resistance of blackleg. The epidemiology of blackleg differs between location and regions because of differences in climate, growing season, cultivars and especially fungal populations [9].

Symptomatology

During the diseases on set, emergence of brown irregular spots on stem base of rapeseed was observed during the second week of August 2018 (Figure 2). Although the increase in the number of spots occurred over time, involving a larger tissue area, delayed growth or decay of the infested plants was not observed. Based on the visual observation, only the necrosis of surface tissue was detected during August to October [10-12].

Leptosphaeria maculans were found attacking nearly all parts of the plant, including cotyledons, leaves, stems, roots and pods, and cause leaf lesions and stem cankers (Figure 2). The first obvious symptoms of blackleg on leaves were the appearance of gray-green to ash-gray lesions on the lower leaves (Figure 2). The presence of small, black pycnidia at the edge or scattered across the blackleg lesions distinguishes them from lesions caused by another common foliar pathogen, *Alternaria brassicae*. Basal stem lesions are the most

Table 1: Survey location and blackleg disease distribution.

Location	N	E	Altitude (m.a.s.l.)	Black leg severity (%)	Variety
Kulumsa	08°01'09.169	039°09'18.921	2176	0	<i>B. carinata</i>
Eteya 1	08°01'06.439	039°09'17.466	2174	0	<i>B. carinata</i>
Kulumsa seed enterprise	08°02'28.772	039°11'49.197	2262	0	<i>B. carinata</i>
Eteya 2	08°05'45.400	039°13'25.284	2200	0	<i>B. carinata</i>
Eteya 3	08°06'31.492	039°13'38.069	2207	0	<i>B. carinata</i>
Robe gebeya	09°12'48.318	038°27'36.295	2898	0	Dual
Cheri	09°21'20.700	038°46'15.450	2892	0	Dual
Robe gebeya	09°21'09.570	038°46'21.920	2855	0	Dual
Robe gebeya (Agota)	09°20'59.700	038°46'13.180	2853	0	Dual
Holetta on station 1	09°03'23.441	038°30'07.492	2366	25.6	Rape seed
Holetta zuria 1	09°07'52.076	038°26'18.706	2614	3.5	<i>B. carinata</i>
Holetta zuria 2	09°05'37.760	038°27'14.420	2536	5.5	<i>B. carinata</i>

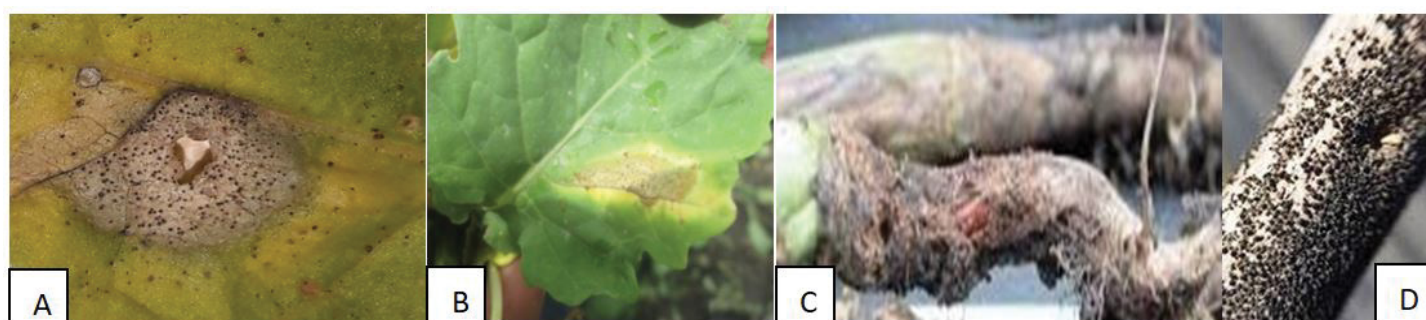


Figure 2: Symptom of black leg on leaf A & B, symptoms on stem with pycnidia, C & D.

damaging. When these occur in the seedling or rosette phases of growth, symptoms resemble damping-off or cut-worm damage. In older plants, the more canker symptom leads to premature ripening or lodging (falling over) of the crop. Based on the cross-section and longitudinal section of infested plants during the second half of the growing period, further decay of infested tissue was detected. Besides delayed growth, infested plants had fewer side stems and flowers during inflorescence, which resulted in yield decrease.

Morphological characteristics

From samples collected, *L. biglobosa* (B group isolate) were frequently isolated majorly from leaf 52.08%; whereas *L. maculans* were from cankered stem and leaf (31.25%) and *Alternaria* were also isolated (16.7%) from leaf. From the study we confirm that, the presence of the aggressive pathogen *L. maculans* and weakly virulent pathogen *L. biglobosa* in major producing areas and different *brassica* species. The result supported by Williams and Fitt [13], Strains of *L. maculans* were classified into two pathotypes: the highly virulent, aggressive 'A' group strains that cause stem cankers, and the nonaggressive, weakly virulent, 'B' group strains that do not cause stem cankers. According to Johnson & Lewis, A group isolates caused damaging brown, cortical lesions but B group isolates penetrated the leaf gap to enter the stem pith, rarely causing externally visible phoma stem canker (Table 2).

Cultural characteristics

Colonies were found circular in shape grew on a PDA medium after 5 days (at 25°C ± 1°C), and were observed in isolates: BLHH-1, BLHH-2, BLHH-3, BLHH-4, LM-1, LM-2, LB-1, and LB-2. Mycelia were loose, colored white to white smoke. Some of them form colonies with irregular round shape and lobular edges after 5 days (Figure 3).

The pycnidia of the fungus were black, globose to subglobose in shape, the single-celled conidia, hyaline and fusiform with diameters of 4–5 × 1.5–2 µm. Most of the isolates were produced pigment in the liquid culture in variable color brown to black (Figure 4).

Growth rate

From the result slow growth was observed on Isolate (BLHH-1, BLHH-2, BLHH-3, BLHH-4, LM-1 AND LM-2) with high sporulation, which is a typical characteristic of *L. maculans* of A group isolate, whereas faster growth rate was observed on isolate (LB-1 AND LB-2) with low sporulation which is the characteristics of *L. biglobosa* (Table 3).

Pigment formation on liquid Czapek agar

For the purpose of isolates separation, based on pigment formation on liquid Czapek agar, it was observed that after 30 days isolates LM-1, LM-2, LB-1 AND LB-2 where produce yellow-brown pigment which indicate places isolates in a group of non-aggressive strains in conformity with the *L. biglobosa*. Isolate BLHH-1, BLHH-2, BLHH-3 and BLHH-4 which did not produce pigment; the situation indicates the aggressiveness of the isolate and which is under group *L. maculans* (Figure 5). The pathotypes are visually indistinguishable in culture but can be differentiated on their ability to produce pigment in liquid culture, colony growth rate, and disease reactions on *B. napus* [14].

SUMMARY AND RECOMMENDATIONS

From the survey based on morphological and cultural characterization of the isolates two types of phytopathogenic fungi of *Leptosphaeria* complex: *Leptosphaeria maculans* and *Leptosphaeria biglobosa* were found. As a result we recommend the breeding program need to consider both isolates in their breeding program. The isolation relied on different pigment production in media to distinguish A group and B group isolates. This study also needs to support by molecular means of characterization. The study covers and includes isolates from only few producing areas, in order to know the status of the pathogen in the country, major rapeseed producing areas is required further assessment. Where *L. maculans* populations are a mixture of A group and B group, the proportions of the different isolates appear to be influenced by a complex interaction between pathogen, host, climatic and cultural

Table 2: Isolation frequency.

S. No	Species	No. of isolate		Total	(% age)
		Leaf	Stem		
1	<i>L. maculans</i>	10	5	15	31.25
2	<i>L. biglobosa</i>	25	0	25	52.08
3	<i>Alternaria</i>	8	0	8	16.70
Total		43	5	48	100

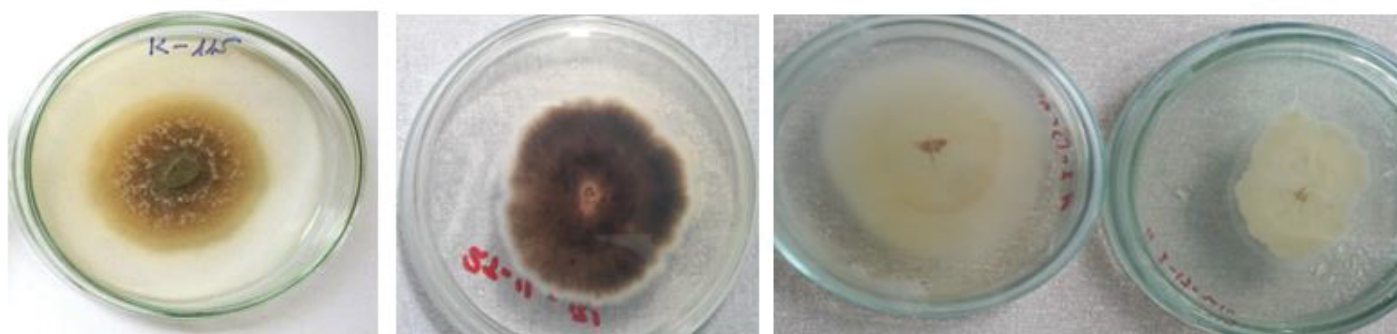


Figure 3: Colony of the isolate on PDA-1.

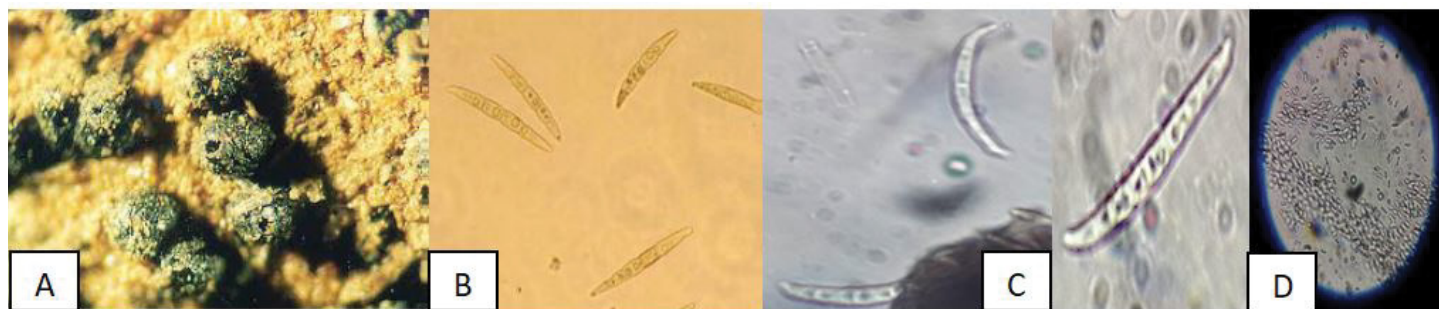


Figure 4: Colony of the isolate on PDA-2.

Table 3: Diameter (cm) and sporulation of tested isolates on a PDA at $25 \pm 1^\circ\text{C}$.

Isolates	After 5 days	Sporulation	After 10 days	Sporulation	After 15 days	Sporulation
BLHH-1	0.57	-	1.27	++	2.07	+++
BLHH-2	0.95	-	1.97	++	2.6	+++
BLHH-3	2.03	-	2.87	-	3.23	-
BLHH-4	1.53	-	2.47	-	2.80	-
LM-1	1.53	-	3.37	++	5.21	+++
LM-2	1.49	-	3.59	++	4.96	+++
LB-1	1.61	-	3.94	-	8.9	-
LB-2	1.56	-	4.32	-	9	-

-No sporulation + Weak sporulation ++ Medium sporulation +++ Strong sporulation

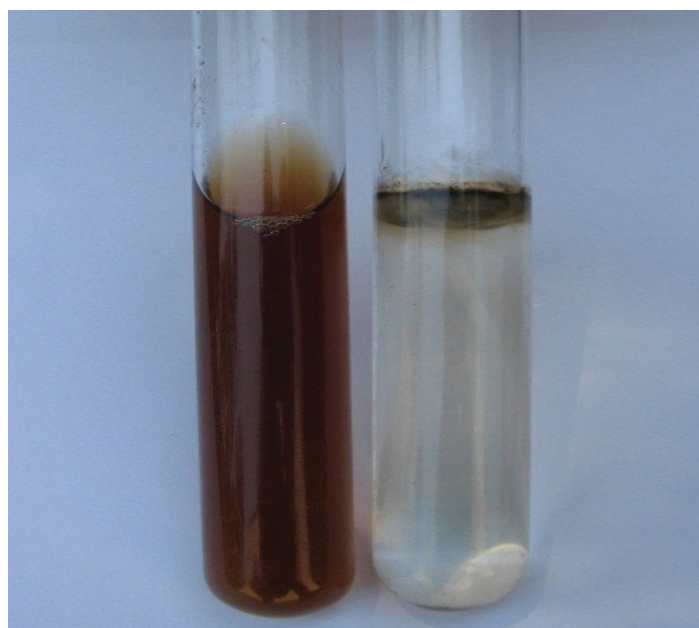


Figure 5: Colony of the isolate on PDA-3.

factors. Even within a region, there can be large differences in the population structure of the pathogen.

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